

**Investigating the Effect of Temperature on Pump-Driven Antibiotic  
Resistance towards Erythromycin in *Escherichia coli***

Submitted by

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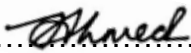
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## Abstract

Antibiotic resistance is an inevitable by-product from treatment of bacterial (and fungal) infections, however the rate and intensity at which it is emerging is alarming. More and more drugs are being rendered ineffective, where poor treatment behaviours, such as overuse, are being held accountable. Coupled with a dry antibiotic pipeline, we are increasingly seeing ourselves approach a post-antibiotic era. As research is being conducted into discovering new antibiotics, we also need to find ways to preserve the ones we still have. Therefore it is equally important to identify and study potential selection pressures, both environmental and clinical, that contribute to the rise in resistance.

To provide a preliminary insight on temperature as a possible selection pressure, this research project aimed to investigate the effect of temperature on the susceptibility of *Escherichia coli* to the macrolide-class antibiotic erythromycin, and ultimately its effect on the expression of multidrug efflux pump AcrAB-TolC. This was done by exposing *E. coli* cells to a range of erythromycin concentrations during 24 hours of growth, establishing a minimum inhibitory concentration (MIC) at 30°C and seeing any shifts in the MIC at 37°C. It was found that as temperature increased from 30°C to 37°C, so did the MIC. Thus the cells were more resistant at the higher temperature.

Next, to see whether and how AcrAB-TolC was selected for as a result of temperature, the expression of protein AcrB was measured via fluorescence emitted from sfGFP (due to the *sfGFP* gene being physically fused to the *acrB* gene). It was found that the range of concentrations that select for pump expression, referred to as the “AcrB expression-selection window”, shifted positively with increasing temperature. This suggests a temperature-dependent nature of resistance selection, therefore this knowledge may help in choosing an effective dose for treatment based on thermal conditions.

The outcomes of this research project will help provide a foundation for looking further into temperature, and other selection pressures, and their effect on the rise in antibiotic resistance.



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## Contents

<b>Abstract.....</b>	<b>3</b>
<b>Acknowledgements.....</b>	<b>5</b>
<b>List of Figures.....</b>	<b>9</b>
<b>List of Tables.....</b>	<b>12</b>
<b>List of Abbreviations.....</b>	<b>14</b>
<b>Chapter 1: Introduction.....</b>	<b>15</b>
1.1 Mechanisms of Resistance.....	18
1.1.1 Efflux Pumps.....	21
1.2 AcrAB-TolC.....	23
1.3 Erythromycin.....	25
1.4 Examples of Selection Pressures.....	27
1.4.1 Low Environmental Antibiotic Concentrations.....	27
1.4.2 Motility and Environmental Heterogeneity.....	28
1.4.3 Heavy Metals.....	28
1.4.4 Temperature.....	29
1.5 Project Aims.....	32
<b>Chapter 2: Obtaining OD-to-CFU/ mL Conversion Factors and Investigating the Effect of Temperature on the Growth Profiles of <i>E. coli</i> Strains MG1655, eTB108 and AG100-A.....</b>	<b>34</b>
2.1 Materials and Methods.....	34
2.1.1 Strains.....	34
2.1.2 Media.....	35
2.1.3 Growth Curve Protocol.....	36
2.1.4 Obtaining OD-to-CFU/ mL Conversion Equations.....	36
2.2 Results.....	40
2.2.1 A Comparison of Growth Curves between Strains.....	40
2.2.2 A Comparison of Growth Curves between Temperatures...42	
2.2.3 OD-to-CFU/ mL Conversion.....	45
2.2.3.1 Conversion Factor per Time Point.....	45
2.2.3.2 Mean OD-to-CFU/ mL Conversion Factors and Standardising the Inoculum Size.....	48

2.3 Discussion.....	50
<b>Chapter 3: The Effect of Temperature on the MIC of MG1655, eTB108 and AG100-A, and on the Selection of Pump Expression.....</b>	<b>54</b>
3.1 Materials and Methods.....	54
3.1.1 Erythromycin Stock.....	54
3.1.2 Plate Reader Setup.....	54
3.1.3 MIC Assay.....	55
3.2 Results.....	58
3.2.1 Growth Rate Increases with Temperature.....	58
3.2.2 MIC Increases with Temperature.....	61
3.2.3 Selective Doses for Pump Expression Shift with Temperature.....	63
3.3 Discussion.....	65
<b>Chapter 4: Concluding Remarks and Future Directions.....</b>	<b>69</b>
<b>Supplementary.....</b>	<b>71</b>
<b>References.....</b>	<b>84</b>



## List of Figures

Figure 1.1: A schematic of the four mechanisms of antibiotic resistance within a bacterial cell.

Figure 1.2: An illustration of the five major families of efflux pumps found in the prokaryotic kingdom.

Figure 1.3: A) A pseudo-atomic model of the structure and arrangement of the efflux pump AcrAB-TolC in association with AcrZ and B) A diagram representing the hypothesised mechanism by which substrates are extruded by the efflux pump into the extracellular environment with the aid of the proton motive force.

Figure 1.4: Schematic of a macrolide molecule e.g. erythromycin bound to the peptide exit tunnel. The nascent protein is blocked from exiting the ribosome.

Figure 2.1A: A schematic of the general growth curve protocol used in this study for each strain and experimental temperature.

Figure 2.1B: A schematic of the protocol used to measure OD and CFU/ mL of a series of 2-fold dilutions for the 6<sup>th</sup>, 8<sup>th</sup>, 24<sup>th</sup>, 26<sup>th</sup>, 28<sup>th</sup> and 30<sup>th</sup> hour of culture growth.

Figure 2.2: Growth curves over time for *E. coli* strains MG1655 (WT, blue), eTB108 (*acrB-sfGFP*, orange) and AG100-A ( $\Delta$ *acrAB*, grey) at 30°C.

Figure 2.3: Growth curves over time for *E. coli* strains MG1655 (WT, blue), eTB108 (*acrB-sfGFP*, orange) and AG100-A ( $\Delta$ *acrAB*, grey) at 37°C.

Figure 2.4: Growth curves over time for *E. coli* strain MG1655 (WT) at 30°C (blue) and 37°C (red).

Figure 2.5: Growth curves over time for *E. coli* strain eTB108 (*acrB-sfGFP*) at 30°C (blue) and 37°C (red).

Figure 2.6: Growth curves over time for *E. coli* strain AG100-A ( $\Delta acrAB$ ) at 30°C (blue) and 37°C (red).

Figure 2.7: OD-to-CFU/ mL conversion factor over time for *E. coli* strain MG1655 (WT) at 30°C (blue) and 37°C (red).

Figure 2.8: OD-to-CFU/ mL conversion factor over time for *E. coli* strain eTB108 (*acrB-sfGFP*) at 30°C (blue) and 37°C (red).

Figure 2.9: OD-to-CFU/ mL conversion factor over time for *E. coli* strain AG100-A ( $\Delta acrAB$ ) at 30°C (blue) and 37°C (red).

Figure 2.10: Average OD-to-CFU/ mL conversion factor for *E. coli* strains MG1655 (WT), eTB108 (*acrB-sfGFP*) and AG100-A ( $\Delta acrAB$ ) at 30°C (blue) and 37°C (red).

Figure 3.1: The overall protocol used to inoculate microplates with MG1655, eTB108 or AG100-A to determine MICs and measure FI using plate reader.

Figure 3.2: Growth curves over time for *E. coli* strain MG1655 (WT) at 30°C (blue) and 37°C (red) using plate reader.

Figure 3.3: Growth curves over time for *E. coli* strain eTB108 (*acrB-sfGFP*) at 30°C (blue) and 37°C (red) using plate reader.

Figure 3.4: Growth curves over time for *E. coli* strain AG100-A ( $\Delta acrAB$ ) at 30°C (blue) and 37°C (red) using plate reader.

Figure 3.5: Mean growth rate per minute for *E. coli* strains MG1655 (WT), eTB108 (*acrB-sfGFP*) and AG100-A ( $\Delta acrAB$ ) at 30°C (blue) and 37°C (red).

Figure 3.6: Dose-response curves for *E. coli* strains MG1655 (WT, blue) and eTB108 (*acrB-sfGFP*, orange) at 30°C (circle) and 37°C (triangle).

Figure 3.7: Dose-response curves for *E. coli* strain AG100-A ( $\Delta acrAB$ ) at 30°C (blue) and 37°C (red).

Figure 3.8: Relative expression levels of AcrB per cell as a function of erythromycin dose of sfGFP-tagged *E. coli* strain eTB108 (*acrB-sfGFP*) at 30°C (blue) and 37°C (red).

Figure S1: Regressions of mean OD vs log CFU/ mL for *E. coli* strain MG1655 (WT) at 30°C per time point.

Figure S2: Regressions of mean OD vs log CFU/ mL for *E. coli* strain MG1655 (WT) at 37°C per time point.

Figure S3: Regressions of mean OD vs log CFU/ mL for *E. coli* strain eTB108 (*acrB-sfGFP*) at 30°C per time point.

Figure S4: Regressions of mean OD vs log CFU/ mL for *E. coli* strain eTB108 (*acrB-sfGFP*) at 37°C per time point.

Figure S5: Regressions of mean OD vs log CFU/ mL for *E. coli* strain AG100-A ( $\Delta acrAB$ ) at 30°C per time point.

Figure S6: Regressions of mean OD vs log CFU/ mL for *E. coli* strain AG100-A ( $\Delta acrAB$ ) at 37°C per time point.

## List of Tables

Table 1.1: Amino acid substitutions in residue 288 of AcrB in MDR strains of *Salmonella* isolated from a patient at different times during antibiotic therapy.

Table 2.1: *Escherichia coli* K-12 derivative strains used in this study.

Table 2.2: Recipes for all media used throughout study.

Table 2.3: Stock solutions used to create M9 media.

Table 2.4: Mean OD-to-CFU/ mL conversion factor per strain and temperature.

Table S1:  $p$ -values for Fig. 2.2 (30°C growth curves) obtained from unpaired t-test.

Table S2:  $p$ -values for Fig. 2.3 (37°C growth curves) obtained from unpaired t-test.

Table S3:  $p$ -values for Fig. 2.4 (MG1655 growth curves) obtained from unpaired t-test.

Table S4:  $p$ -values for Fig. 2.5 (eTB108 growth curves) obtained from unpaired t-test.

Table S5:  $p$ -values for Fig. 2.6 (AG100-A growth curves) obtained from unpaired t-test.

Table S6:  $p$ -values for Fig. 2.7 (MG1655 OD-to-CFU/ mL conversion factors) obtained from unpaired t-test

Table S7:  $p$ -values for Fig. 2.8 (eTB108 OD-to-CFU/ mL conversion factors) obtained from unpaired t-test.

Table S8:  $p$ -values for Fig. 2.9 (AG100-A OD-to-CFU/ mL conversion factors) obtained from unpaired t-test.

Table S9:  $p$ -values for Fig. 2.10 (mean OD-to-CFU/ mL conversion factors) obtained from unpaired t-test.

Table S10: OD-to-CFU/ mL conversion factors (CF) and  $R^2$  for each time point, per strain, per temperature

## **List of Abbreviations**

WHO: World Health Organisation

EU: European Union

CDC: Centre for Disease Control

ECDC: European Centre for Disease Control

RPP: Ribosomal Protection Protein

MDR: Multidrug Resistance/ Multidrug Resistant

ATP: Adenosine Triphosphate

ABC: Adenosine Triphosphate Binding-Cassette

MATE: Multidrug and Toxic Efflux/ Multidrug and Toxic compound Extrusion

MFS: Major Facilitator Superfamily

SMR: Small Multidrug Resistance

RND: Resistance Nodulation and cell Division

NPC1: Niemann-Pick C1

NPC: Niemann-Pick type C

rRNA: Ribosomal RNA

MIC: Minimum Inhibitory Concentration

MSC: Minimum Selective Concentration

LB: Luria-Bertani

HSP: Heat Shock Protein

CSP: Cold Shock Protein

CAMP: Cationic Antimicrobial Peptide

WT: Wild-Type

sfGFP: Superfolder Green Fluorescent Protein

FI: Fluorescence Intensity

w/v: Weight/Volume

OD: Optical Density

CFU: Colony Forming Unit

GFP: Green Fluorescent Protein

CF: Conversion Factor

## Chapter 1 – Introduction

Antibiotics, considered one of the greatest discoveries in the history of modern medicine, are a group of drugs used for treating infections caused by bacteria as well as fungi. First discovered in the 1940s, their misuse, such as being widely prescribed and over-used in treatment and in maintaining livestock, has contributed to the rapid emergence and spread of resistant strains of bacteria globally [1, 2, 3]. This is termed as antibiotic resistance, a current pandemic that, according to the World Health Organisation (WHO), needs to be a high priority in research to prevent disruption on progress in public health and food security [1]. As a consequence, this increased rate of emergence has led to longer durations of hospitalisation, higher medical costs and mortality rates, as even common infections are becoming increasingly difficult to treat [1, 2, 3]. For instance, a study conducted in the European Union (EU) found that between 2007 and 2015, the average incidence of infections caused by a selected group of resistant bacteria (commonly found in blood diseases) increased from 239,288 to 602,609 cases. In 2007, approximately 25,000 deaths and 2.5 million extra hospital days were attributed to the resistant infections, contributing to an overall extra medical cost of EUR 900 million. In 2015, however, the number of deaths increased to 33,000 [4, 5].

It should be noted that antibiotics have always existed in nature, produced by some microbes as a means of subduing competitors in an ecological niche, the same compounds that we exploit today in the clinic. As a result of the existence of this artillery in the environment, many competitors have evolved resistance over time to tackle the toxic compounds. This particular type of pre-existing antibiotic resistance is known as intrinsic resistance [6, 7]. However, the type that is of major public concern nowadays is acquired resistance. As the name suggests, this is when previously-susceptible cells acquire defence mechanisms by either taking up resistance-containing genetic material e.g. plasmids from other bacterial cells or the environment, or undergoing a spontaneous mutation or overexpressing a particular gene(s) as a response to a selection pressure [7]. Poor treatment behaviours such as over-use are one of many factors contributing to speeding up the process as well as amplifying the level of acquired resistance [1, 2, 3, 4].

To tackle the rate at which antibiotic resistance is emerging, plans of action have been suggested by organisations such as WHO, the Centre for Disease Control (CDC) and the European Centre for Disease Prevention and Control (ECDC) along with scientists established in the field of drug resistance. The general consensus agreed on measures such as public education on behaviours that lead to resistance, prevention strategies, strict control over antibiotic use and looking into finding new antibiotics and/or alternatives (e.g. antibacterial vaccines) [1, 4, 8, 9]. The discovery of new antibiotics, however, has proven to be significantly difficult, and thus no new class has been introduced in the clinic in the last few decades. This is mainly due to exhausting the available resources from where antibiotics can be cultivated, such as soil [10, 11]. Ultimately, this has led to the rate of the loss of effective antibiotics (as a result of developing resistance) surpassing the rate of novel antibiotic discovery. In other words, this slow and limited supply of antibiotics is struggling to meet the increasingly high demand for them [12]. Only relatively recently has the potential antibiotic, termed “teixobactin”, been discovered, but it still has yet to undergo clinical trials before its release to the wider public [12, 13].

Another critical concern is that any new antibiotic discovered will inevitably suffer the same fate of resistance development befallen to previous ones. Therefore, not only is discovering new antibiotics important, but the way we use our current and future ones is equally just as important. Along with present-day implemented control actions such as using narrow-spectrum antibiotics during treatment (as opposed to broad-spectrum) that are more specific to the causal pathogen, and opposing unnecessary prescribing of antibiotics in the clinic [2, 3, 8, 9], we need to find better and more effective ways to preserve the ones we already have. This can only be achieved by studying and defining selection pressures that lead to the increased rate of resistance.

Generally speaking, selection pressures, as the name suggests, are conditions or factors that when applied select for a favourable trait and gradually its fixation within a population i.e. the trait becomes more common over time. Charles Darwin explained in his publication “On the Origin of Species” that when exposed to such pressures, the variations in characteristics observed within a population play a critical part in survival [14]. Those individuals that have a more



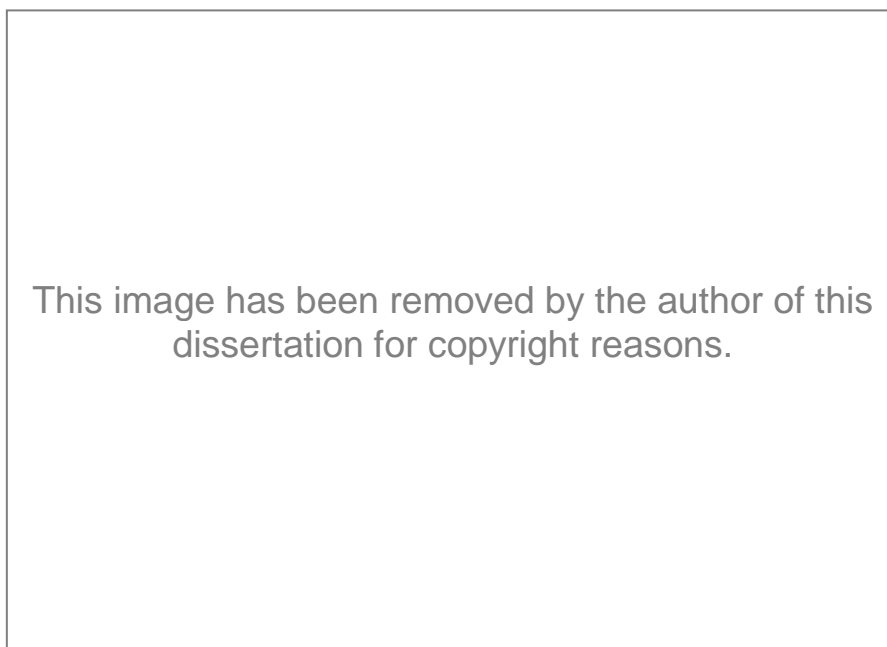
favourable phenotype (or genotype) to the current environmental condition may survive, reproduce and pass on such traits to their progeny, whilst the individuals with the least desirable traits will be, according to Darwin, “rigidly destroyed” by the change in condition i.e. the ill-suited phenotype will gradually cease to exist. It is this phenomenon, termed “natural selection”, which leads to the evolution of species [14].

Although based on animals and plants, Darwin’s observations can also be seen amongst bacteria, a prime example being acquired resistance in the clinic. During a course of treatment, the bacterial population is exposed to a harsh antibacterial environment. Amongst the cells, individuals that express some form of resistance to the drugs used during the treatment (as a result of accumulating random mutations and/ or genetic material) are able to reproduce and pass on this favourable genotype. With prolonged exposure including attempts to treat with higher doses, amplification in the level of resistance and the genotype fixating itself within the population may eventually occur, gradually rendering the treatment drug(s) useless [15].

As mentioned previously, the use of antibiotics in treatment is just one of several other selection pressures that come together to play a part in the observed increased rate of emerging resistance seen globally. Some of these potential pressures, i.e. exposure to low concentrations of antibiotics that exist within the environment, motility in a spatially complex environment and the presence of other toxic compounds will be looked into further in this chapter, with special emphasis on temperature throughout the report as this is the main variable in this research project.

## 1.1 Mechanisms of Resistance

There are different types of mechanisms bacteria can deploy to protect themselves against antibiotics ([Fig. 1.1](#)).



**Figure 1.1:** A schematic of the four mechanisms of antibiotic resistance within a bacterial cell taken from reference [16](#). Efflux pumps, shown in the top left corner (purple), are the main topic of this research.

One method is by enzymatically altering or degrading the drug molecule so that it can no longer bind to its target in the bacterium [[7](#), [17](#)]. An example of this is seen in penicillin resistance provided by  $\beta$ -lactamase enzymes [[18](#), [19](#)]. These enzymes render  $\beta$ -lactam antibiotics, such as penicillin, ineffective by cleaving a part of the drug. The spread of penicillin resistance was found to be due to  $\beta$ -lactamase-encoding plasmids being transferred between bacteria [[19](#)]. To tackle the action of these enzymes, research is being conducted in three areas: the production of synthetic antibiotics with an altered or removed enzyme-target region, inhibitor molecules to bind and block the enzyme active site, and improving the delivery of drugs to the corresponding targets in the bacterium [[20](#)].

A second method of resistance involves altering the drug target, either by a mutation occurring in one or more of its associated genes, chemical modification of the components, or protecting the target to prevent access so

that the antibiotic molecule is no longer able to bind and carry out its function [7, 17]. A well-studied example is tetracycline resistance mediated by the ribosomal protection proteins (RPPs) Tet(O) and Tet(M). The antibiotic tetracycline binds to bacterial ribosomes, interfering with protein synthesis and thus preventing microbial growth and viability. To circumvent this, the RPPs, as their name suggests, protect the ribosome by dislodging the bound drug so that protein synthesis can resume [21]. Tet(M) specifically has been found on conjugative transposons, which are mobile genetic elements similar to plasmids but are able to integrate and excise themselves into and out of the bacterial genome. In addition, conjugative transposons are able to be transferred between different bacterial species, contributing to the wide dissemination of antibiotic resistance [22, 23]. One way to overcome target protection is to develop antibiotics that are able to bind more effectively to the target, preventing their removal. In the case of Tet(O) and Tet(M), glycyclines are able to stay bound to the ribosome even in the presence of the RPPs [24].

Another option is the use of combination therapy in which more than one drug are used that a) enhance the potency and action of the antibiotic(s), and/or b) bind to different target sites in the pathogen to overcome the effect of resistance mechanisms [25]. For example, tetracycline is commonly used in combination with the antibiotic metronidazole and other compounds as second-line treatment in *Helicobacter pylori* infections, where both antibiotics work synergistically to eradicate the bacteria [26, 27]. However, it should be noted that in some developing areas the above drug combination may fail, such as in Shanghai in the People's Republic of China, where tetracycline and metronidazole-resistance is prevalent [27, 28]. Interestingly, a study had shown that the genotypes of *H. pylori* varied between isolates all over the globe [29], which may suggest a variation in the selection pressures they are exposed to. This again highlights the importance of studying these conditions in order to tailor specific therapies that are effective in treatment, all whilst reducing the impact on the rate of antibiotic resistance emergence.

A third defence mechanism against antibiotics is by bacteria reducing the permeability of their cell wall so that the drug is not able to gain access to its target [7, 17]. The cell surface of Gram-positive bacteria contain a lipid bilayer membrane coated by a thick layer of peptidoglycan. Lipids in this arrangement

create a hydrophobic layer which many molecules may struggle to bypass, particularly if they are large and/ or hydrophilic like many antibiotics. In addition, Gram-negative bacteria have another lipid bilayer surrounding their relatively-thin peptidoglycan layer, providing an extra level of intrinsic resistance by further restricting the movement of molecules. This outer-membrane also contains protein channels known as porins, allowing the movement of small hydrophilic molecules in and out of the cell [30, 31]. However, the bacterium may reduce the expression of these surface proteins or lose them altogether to further reduce membrane permeability [7, 17, 31]. To tackle this, antibiotics can be coupled with membrane permeabilisers such as polymyxin antibiotics as part of combination therapy to allow penetration of the main drug into the cell and/ or eventual cell lysis [32]. However, polymyxin-resistant strains of *Escherichia coli* and *Salmonella typhimurium* have been isolated [32]. In fact, the polymyxin drug colistin had always been considered as a last-resort antibiotic for multidrug-resistant (MDR) infections. However recently incidences of colistin resistance have risen at an alarming rate in many parts of the world, attributed to a plasmid-mediated spread of the resistance gene [33, 34, 35, 36, 37, 38]. Therefore it is important for future studies to look into how this resistance occurs, and the development of more effective membrane permeabilisers that are less likely to have resistance develop against them.

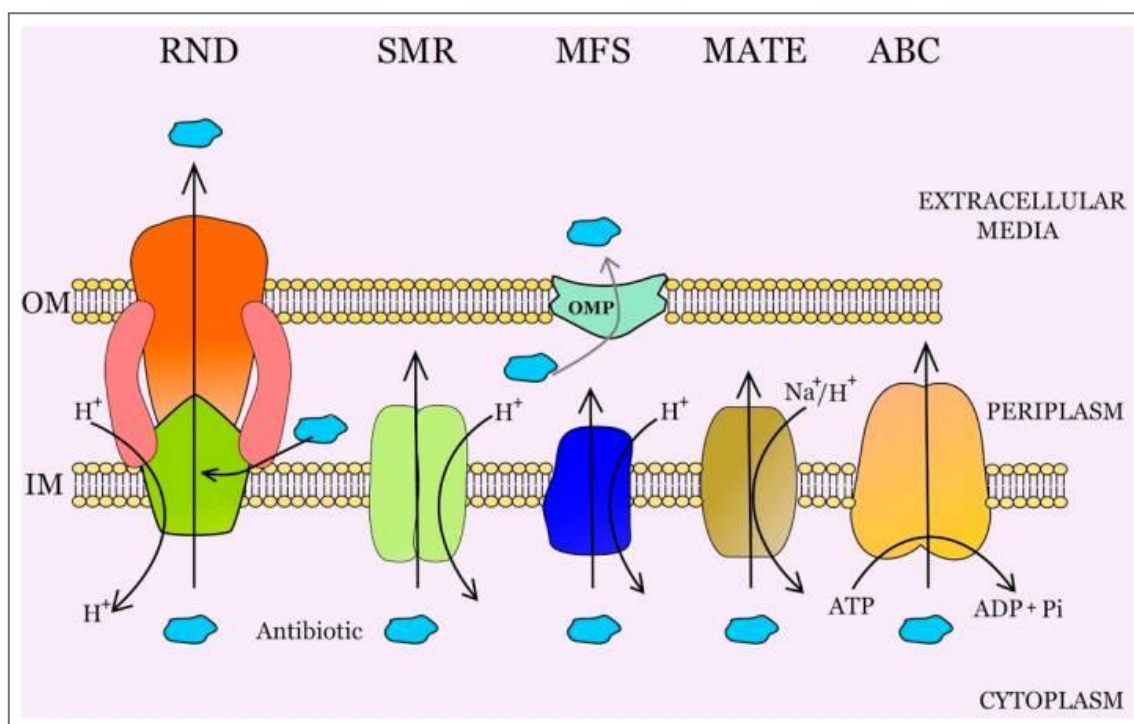
Efflux pumps, the fourth defence mechanism, are a good example of both intrinsic and acquired resistance, as they are constitutively expressed at a basal level in some bacteria, but can be overexpressed as a response to the environment [6, 39]. They are proteinaceous transporters that are found on the cell membrane of different organisms from different domains of life [40, 41], including humans [42, 43] as well as bacteria, with the purpose of expelling toxic substances from within the cell into the environment where it will cause less harm. These toxic substances include those produced by the cell as a result of metabolism, as well as competitor-produced antibiotics [6, 39]. Discovered in the 1980s, the study of efflux pumps is steadily creeping into the forefront of research due to their role in MDR [44, 45].

### 1.1.1 Efflux Pumps

Prokaryotic pumps can be divided into families based on certain criteria such as their structure, components and sequence, the substrates they utilise and their source of energy [44, 46] (Fig. 1.2).

The adenosine triphosphate (ATP)-binding cassette (ABC) superfamily, as the name suggests, depends on ATP as its energy source to actively transport molecules out of the cell [47]. Interestingly, the first reported bacterial ABC efflux pump was found in *Lactococcus lactis*, specifically discovered through homologous-linking with the human ABCB1 efflux pump known to be involved in chemotherapy resistance [48]. The Multidrug and Toxic Efflux (or Multidrug and Toxic compound Extrusion) family (MATE), also found amongst many organisms e.g. NorM in *Vibrio parahaemolyticus*, are a group of Na<sup>+</sup>/ drug antiporters where compounds such as fluoroquinolones are expelled in exchange for Na<sup>+</sup> [49].

The Major Facilitator Superfamily (MFS), one of the largest groups that are mainly found in Gram-positive species, are also antiporters but instead import H<sup>+</sup> ions, and therefore take advantage of the proton motive force to provide energy to export compounds out of the cell [50]. One of the first examples and of clinical relevance is the pump QacAB found in *Staphylococcus aureus*, discovered to be plasmid-mediated through many incidents of highly-resistant hospital-acquired infections [51]. The Small Multidrug Resistance (SMR) family also utilise the energy provided from the movement of protons but are relatively smaller, such as the 110-amino acid long EmrE pump in *E. coli* [52].

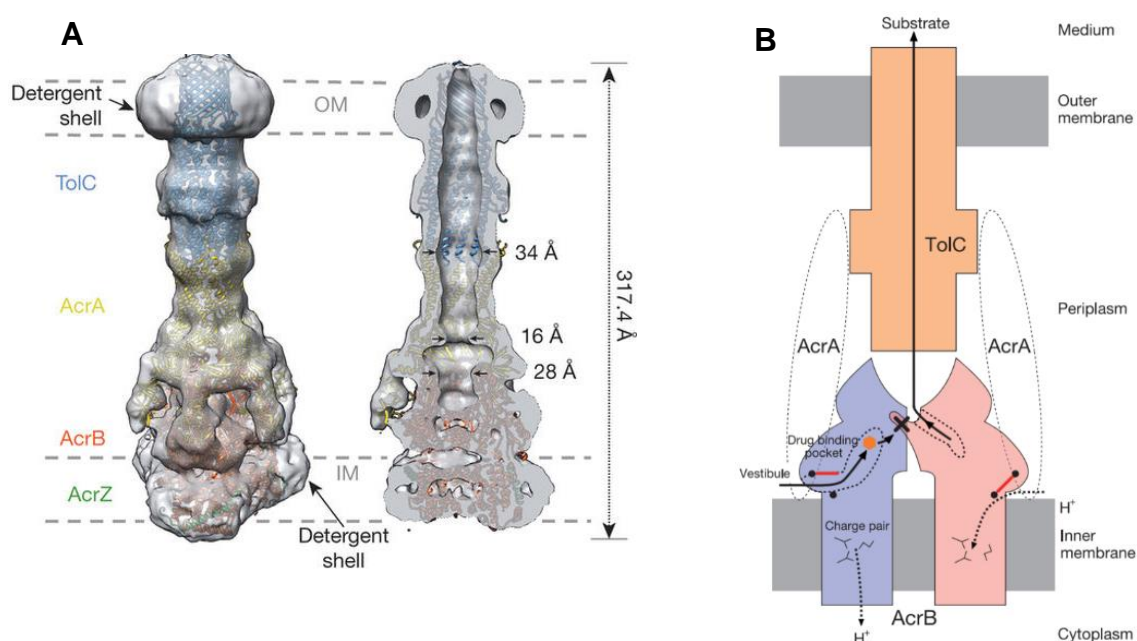


**Figure 1.2:** An illustration of the five major families of efflux pumps found in the prokaryotic kingdom, taken from reference 46. The Resistance Nodulation and cell Division (RND), Small Multidrug Resistance (SMR), Major Facilitator (MFS), Multidrug and Toxic Efflux (MATE) and Adenosine Triphosphate-Binding Cassette (ABC) families differ through structure, energy source and substrate.

Of all the classes, the Resistance Nodulation and cell Division (RND) superfamily, found mostly in Gram-negative bacteria, may be deservedly considered the most extensively studied due to their paramount role in drug-resistant infections caused by clinically-relevant pathogens [44, 45]. Pumps of this group mainly form tripartite complexes, consisting of components in the inner membrane, the periplasmic space and the outer membrane coming together, and using the proton motive force to undergo a conformational change and expel toxic compounds from within the cell [44]. A clinically important example of such an efflux pump, and the specific subject of this research study, is the complex AcrAB-TolC.

## 1.2 AcrAB-TolC

AcrAB-TolC consists of the trimeric inner-membrane RND transporter AcrB, the periplasmic hexamer adaptor protein AcrA, and the trimeric outer-membrane channel TolC [53] (Fig. 1.3A). The substrate binds to a drug-binding pocket within a vestibule formed by AcrA and AcrB in the periplasm, and via a rotational mechanism of the whole protein complex, is actively pumped out of the cell using the energy supplied by the movement of protons across the inner membrane [39, 54] (Fig. 1.3B). In *E. coli*, AcrAB-TolC has also been shown to associate with AcrZ, a small component found to modulate some of the molecules passing through the efflux pump [55] (Fig. 1.3A).



**Figure 1.3:** A) A pseudo-atomic model of the structure and arrangement of the efflux pump AcrAB-TolC in association with AcrZ (taken from reference 53) and B) A diagram representing the hypothesised mechanism by which substrates are extruded by the efflux pump into the extracellular environment with the aid of the proton motive force (taken from reference 54).

Due to its non-specificity, AcrAB-TolC is able to bind to a wide range of substrates with very little similarity between them. Thus this efflux pump is directly attributed to MDR [56]. As well as being constitutively expressed in *E. coli*, AcrAB itself is found across a diverse range of Gram-negative species such as *Salmonella* [57], *Klebsiella pneumoniae* [57, 58], *Enterobacter cloacae* [59] and *Yersinia enterocolitica* [60], where its functionality has remained fairly the same. Homologous complexes related to AcrAB-TolC can also be found coexisting within *E. coli* itself (e.g. CusA-CusB which is involved in heavy-metal



expulsion) [39, 61], as well as in other organisms such as MexAB-OprM in the plant pathogen *Pseudomonas aeruginosa* [57, 62]. A distant relative of transporter component AcrB also exists within the eukaryotic kingdom, known as Niemann-Pick C1 (NPC1), and is involved primarily in cholesterol efflux. In humans, mutations in the *npc1* gene is known to cause Niemann-Pick disease type C (NPC), a rare neurodegenerative condition due to the accumulation of cholesterol and other lipids in the brain, liver and spleen [63].

The role of AcrAB-TolC in MDR and its homology to efflux pumps in other organisms makes the complex a prime target for research. The importance of researching AcrAB-TolC has been demonstrated from recent studies. Although some of these studies have shown mutations occurring in AcrAB-TolC and levels of expression of the pump increasing with antibiotic exposure at the transcriptional and translational level [64, 65, 66, 67, 68], the morbid reality of AcrAB-TolC was demonstrated by one particular study. It was observed in this study that during a clinically-approved antibiotic treatment course for a *Salmonella* infection, post-treatment *Salmonella enterica* serovar Typhimurium strains isolated from a patient had been shown to contain a single amino acid substitution in the drug-binding pocket of AcrB, in comparison to the pre-treatment strain [66]. Specifically, glycine (G) was substituted for aspartic acid (D) at residue 288 (Table 1.1), altering the affinity for certain drugs such as ciprofloxacin and nalidixic acid, specifically leading to increased efflux of these two molecules. This was shown to occur within 3 weeks of being on ciprofloxacin treatment, where the substitution was maintained even when the treatment course had progressed onto the antibiotics ceftazidime and aztreonam [66].

**Table 1.1:** Amino acid substitutions in residue 288 of AcrB in MDR strains of *Salmonella* isolated from a patient at different times during antibiotic therapy (taken from reference 66). Post-therapy strains (from L12 onwards) show G288D substitution in AcrB in comparison to pre-therapy strain L3 (red box).

Strain	Weeks posttherapy	MIC, µg/mL						AcrB residue 288
		Cip	Nal	Chl	Tet	Atm	Caz	
L3	0	0.015	2	2	1	0.06	0.12	G
L10	1	0.06	8	8	2	0.12	0.5	G
L11*	3	0.03	16	8	2	0.25	0.25	G
L12	3	0.5	64	32	8	0.5	1	D
L13*	3	0.5	64	16	8	0.5	0.5	D
L6	5	0.5	64	32	8	0.5	0.5	D
L16	17	0.5	64	32	8	0.5	1	D
L18†	19	0.5	64	32	8	0.5	2	D

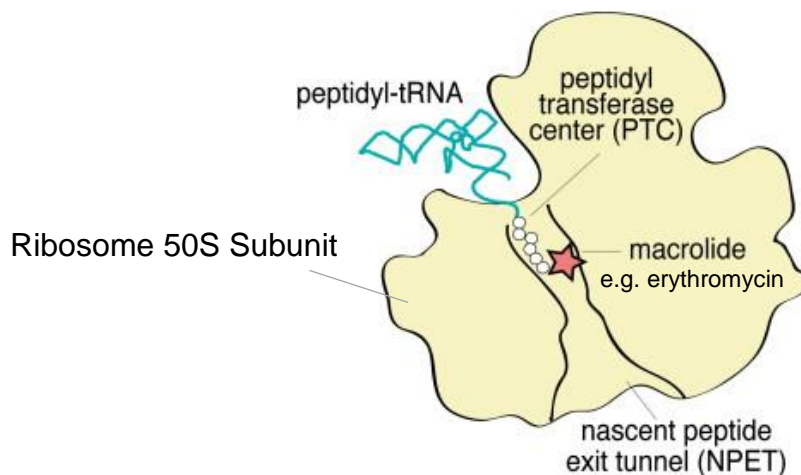


Therefore, in this situation, the presence of a drug selected for the amino acid substitution in order to allow the bacteria to adapt to their hostile environment [66]. It was also believed that due to the substitution, expression of *acrB* doubled in the post-therapy isolates as a consequence. Nonetheless, the amino acid substitution coupled with the increased expression of *acrB* was concluded to have made the infection increasingly difficult to treat, ultimately leading to the patient's death [66, 67].

Another study confirmed, through sequencing of *E. coli* lab strain eTB108 under different doses of the antibiotic erythromycin, that the double expression level can be attributed to the duplication of the *acr* operon (which contains *acrA* and *acrB*). This occurred within as little as three days under drug exposure [68].

Due to its bacteriostatic nature and being a substrate of AcrAB-TolC [68, 69, 70], erythromycin was used as the antibiotic of choice in this research project to induce pump expression.

### 1.3 Erythromycin



**Figure 1.4:** Schematic of a macrolide molecule e.g. erythromycin bound to the peptide exit tunnel. The nascent protein is blocked from exiting the ribosome. Image taken and adapted from reference 69.

Erythromycin, a macrolide-class antibiotic discovered in 1952 [71], is used in the treatment of a range of ailments and major illnesses, from acne to pneumonia and sexually transmitted infections [72]. A clinically relevant translational inhibitor that is effective against Gram-positive as well as Gram-negative bacteria (and a few other non-bacterial pathogens), it acts by reversibly binding to the 23S ribosomal RNA (rRNA) in the larger 50S subunit of the ribosome, blocking the exit of the polypeptide (Fig. 1.4). This causes interference with elongation of the protein chain and thus the release of a truncated polypeptide, where higher concentrations of erythromycin can lead to a bactericidal effect [73, 74].

The first reported case of erythromycin resistance was discovered in the UK in 1959, only a few years after discovery of the antibiotic [75]. As cases rose in other parts of the world [76, 77], a study in Finland determined a positive correlation between frequent erythromycin use in the clinic and the rate of incidence of resistant infections [77]. It should be noted that as well as efflux, erythromycin resistance is also mediated by modification of the ribosome [78], which is beyond the scope of this research project.

To overcome the resistance provided specifically by efflux pumps in clinical treatment, three possible steps were suggested in a research article: 1) remodelling old antibiotics to reduce their affinity to the binding sites of the efflux pump 2) increasing antibiotic uptake by modifying the membrane barrier and reducing efflux through channel blockers and 3) wasting energy sources for efflux with the use of compounds that interfere with energy production [79]. However, the first step may prove to be difficult. This is because evidence has shown, and as stated previously, new antibiotics may eventually succumb to resistance. Although old drugs are being remodelled, the aim of remodelling is to create a new structure to overcome resistance mechanisms. It is therefore highly likely that resistance will also develop against the transformed antibiotics as a result of clinical and environmental factors. In other words, through a lack of understanding of these factors, step 1 would prove useless. The same may also be said for the use of channel blockers as stated in step 2. It is important, firstly, to identify selection pressures and how they come together to optimally select for resistance. This knowledge can then be used to guide antibiotic design in order to treat patients successfully all whilst maintaining the efficacy of

current and future antibiotics and reducing the footprint on resistance evolution. Examples of possible selection pressures that may influence the emergence of antibiotic resistance are discussed below.

## 1.4 Examples of Selection Pressures

### 1.4.1 Low Environmental Antibiotic Concentrations

One of the factors that has been identified as a potential selection pressure is the low-level presence of antibiotics that exist within the environment [80, 81]. Antibiotics can end up in the environment in many ways; through agricultural use and unavoidably human urine and faeces [82]. This presence has been shown to be an insidious force whereby through the selection of low-level resistance, compensatory mutations may occur to reduce the fitness cost over time, eventually leading to high-level clinical resistance [80].

One particular study showed that even at extremely low antibiotic concentrations (similar to those found in aquatic environments and soil) *E. coli* and *S. typhimurium* resistant mutants were selected for within their own mixed population of resistant and wild-type cells [81]. Specifically, for antibiotic concentrations ranging below the minimum inhibitory concentration (MIC), that is, the lowest concentration of antibiotic that inhibits bacterial growth, the research aimed to deduce a selection coefficient i.e. the strength of selection on resistant mutants. Strength was quantified by the change in ratio of resistant: susceptible cells over 80 generations of growth. In other words, the greater the increase in ratio over time, the higher the selection coefficient. When the selection coefficient was zero, the study defines the corresponding antibiotic dose as the minimum selective concentration (MSC) i.e. the lowest concentration at which the resistant mutant is selected due to the fitness cost being balanced by the selective force of the antibiotic concentration [81]. In a mixed population of wild-type and tetracycline-resistant *S. typhimurium* cells, the MSC was found to be 15 ng/ mL of tetracycline, 1/100 of the MIC (1.5 µg/ mL). In the case of *E. coli* and ciprofloxacin resistance, the MSC was 100 pg/ mL for ciprofloxacin, 1/230 of the MIC (0.023 µg/ mL). In addition, it was observed that within a population of only wild-type *S. typhimurium* exposed to  $\frac{1}{4}$

of the MIC associated with streptomycin, *de novo* resistant mutations occurred and proliferated within 200 – 400 generations [81].

#### 1.4.2 Motility and Environmental Heterogeneity

In a complex environment, such as the highly-compartmentalised human body, antibiotic levels are not always uniform, therefore selective concentrations may also exist after antibiotic consumption. Gradients tend to exist, where concentrations decrease when moving further away from the site of drug deployment [83]. A study used a microfluidic device in order to mimic such spatially heterogeneous environments [84]. This device contained several wells attached by small tunnels placed in a ciprofloxacin gradient created using Luria-Bertani (LB) broth as the nutrient source. The study found that *de novo* ciprofloxacin-resistant mutants arose from as little as 100 wild-type *E. coli* cells when they were placed in the antibiotic gradient. It was seen that mutant bacteria were able to quickly move to wells of higher ciprofloxacin where they fixated and divided. These specific points were referred to as “Goldilocks points” as they contained the optimal environment for resistance (i.e. fewer bacterial cells and thus a high nutrient concentration) [84]. Similar results have been seen in chemotherapy resistance [85].

#### 1.4.3 Heavy Metals

Not always does antibiotic resistance and its spread arise as a result of direct antibiotic exposure, but can also occur due to the presence of other toxic compounds and heavy metals [86, 87, 88]. This is due to either the co-localisation of the antibiotic resistance genes and toxin-efflux genes, or cross reactivity of the resistance mechanism itself where it is able to bind to non-antibiotic molecules [89]. In fact, a study had shown that AcrAB increases in expression in the presence of bile-salts and fatty acids, compounds that *E. coli* cells commonly encounter in their normal environment of the intestinal tract. Specifically, they found that the compounds bind to *acr* regulator Rob, which in turn causes the upregulation of the efflux pump [90].

One of the least understood environmental conditions and potential selection pressure for antibiotic resistance is temperature, the main variable in this research study.

#### 1.4.4 Temperature

Temperature variations occur everywhere, from one place to another around the globe, to changes in season and even in different areas of the human body [91, 92]. Climate change i.e. the shift in global weather patterns as a result of forces including greenhouse gases and aerosols, has led to consequences such as increased temperatures, sea levels and changes in rainfall patterns, and has affected not only the environment but the organisms that reside within it [93, 94]. According to WHO, the effects of climate change, such as increased rainfall, are contributing to the spread of disease [95]. Only until very recently, however, have the direct effects of the rise in temperature on antibiotic resistance been noticed. Using a large database containing records on isolates of pathogens *K. pneumoniae*, *E. coli* and *S. aureus* in different parts of the US and mapping them to the local climate, a study showed that regions of higher temperature and population density were linked to a higher fraction of pathogens failing to respond to first-line antibiotics. Therefore it is believed that the trend and burden of emerging antibiotic resistance may be underestimated as factors like climate change are not taken into account [96].

Although the authors were not able to elucidate the exact mechanism of the observed increase in incidence of resistance, they provide a few relevant theories and possible explanations. One of them stems from the outcome of a few studies which shows that with temperatures between 25°C – 37°C, particularly at 30°C, the rate of horizontal gene transfer is relatively high [97, 98, 99]. In fact, recent reports of cases of plasmid-mediated resistance have occurred in countries that average around this temperature range [33, 98]. They also suggest that since temperature affects bacterial growth rates [100], this may also lead to the higher incidences of resistant infections [96].

When temperature is increased, the growth rate of bacteria also increases. Once the optimum temperature is reached e.g. 37°C for some human

pathogens such as *E. coli*, the growth rate is at its highest. Thereafter, higher temperatures start to inhibit proliferation [100]. The growth rate of a microbe has been shown to effect the overall gene expression, as some of the machinery required, such as ribosomes and RNA polymerases, have been shown to be growth rate-dependent [101]. In fact, levels of *acrAB* [102] and *tolC* [103] expression have also been shown to be growth-rate dependant, where in slower-growing cells, higher expression levels have been measured.

Growth rate has been shown empirically to affect susceptibility [104, 105]. A study found that slow-growing cells are more sensitive to some ribosome-targeting antibiotics, but this was largely based on how the drugs bound to their target. By manipulating the nutrient level to control the growth rate, it was found that streptomycin and kanamycin, which irreversibly bound to the ribosome, inhibited the growth of slow-growing *E. coli* at lower drug concentrations in comparison to tetracycline and chloramphenicol, which reversibly bound [104]. The opposite was also seen in fast-growing *E. coli*, where tetracycline and chloramphenicol had the most effect. In other words, slow-growing cells were more susceptible to antibiotics that bound irreversibly to the ribosome [104]. Although not a ribosome targeting antibiotic, another study had shown that slower growing *E. coli* cells were, in contrast, less susceptible to  $\beta$ -Lactam antibiotics [105].

It should be noted that, in most of the aforementioned studies, the growth rate was controlled by nutrient levels. It would be interesting to see if growth rate controlled by temperature produces similar outcomes. This research project provides a preliminary insight on the matter.

As well as growth rate and the type of drug, different bacterial species respond differently to temperature variations. Studies have shown that by increasing the temperature, *S. aureus* becomes increasingly sensitive to tetracycline and penicillin [106, 107]. In another experiment it was shown that elevated temperatures, even in the absence of rifampicin, led *E. coli* strains to develop rifampicin resistance [108]. In the case of *S. aureus*, it was because of the loss of resistance genes and for *E. coli*, a mutation in a gene encoding a component of RNA polymerase [106, 107, 108].

Temperature has also been shown to affect many other aspects of the bacterial cell such as metabolism [109], motility [110], mutation rates [111] and cell shape [112].

It should be noted that when bacteria are suddenly exposed to colder or warmer temperatures than their optimal, this is known as cold shock and heat shock respectively. As a result of these temperature extremes, microbes synthesise proteins, termed heat shock proteins (HSP) and cold shock proteins (CSP) (at around 42°C+ and 15°C- respectively in mesophiles) in order to protect themselves [113]. One would assume that there is a fitness cost associated with expressing such genes, and thus in turn affecting the expression of resistance genes. Interestingly, heat shock proteins have been found to actually decrease susceptibility in some studies [114, 115]. For example, a study in cancer cells showed that the resistance of some tumours to chemotherapy were as a result of the increased expression of HSP27 (Heat Shock Protein 27). The exact mechanism is unknown, but the authors speculate that HSPs interact with cell cycle proteins, enhancing cell division [114]. HSPs have also been found to affect the antibiotic susceptibility of bacterial strains. One such example is the HSP ClpL in *Streptococcus pneumoniae*. ClpL has been shown to influence enzymes involved in cell wall construction, resulting in reduced sensitivity to penicillin [115]. However this particular research project will not look at extreme temperatures, but consideration of HSPs and CSPs will be important in any future experiments that may work with such temperature ranges.

Apart from the differential expression of *acr* genes at different growth rates, literature on the effect of temperature on efflux pumps is extremely limited. One study showed that expression of the efflux pump system RosA-RosB, providing *Y. enterocolitica* with resistance to cationic antimicrobial peptides (CAMPs), occurred at room temperature and 37°C, but only in pathogenic strains [116]. Pump expression in non-pathogenic strains was induced only at room temperature, and repressed at 37°C. The researchers were unable to explain the repression empirically but hypothesise that RosA-RosB may have another role other than providing CAMP resistance in non-pathogenic *Y. enterocolitica* [116]. Therefore it would be interesting to see what the relationship of AcrAB-TolC expression is with temperature.



## 1.5 Project Aims

Based on the aforementioned studies, it is hypothesised that an increase in temperature will have an effect on the susceptibility of bacteria to antibiotics. Therefore, the overall aim of this research project is to investigate the effect of increasing temperature on pump-mediated antibiotic resistance displayed by *E. coli* towards erythromycin. To test this hypothesis, relevant strains of *E. coli* will be exposed to a range of doses of erythromycin which is an AcrAB-TolC substrate, and to temperatures of 30°C and 37°C. These temperatures fall in the thriving range of *E. coli* and many other human pathogens.

In this particular study, resistance will be defined in a scalar manner as an increase in the MIC in comparison to the wild-type MIC (or any other reference MIC), a concept that is widely used in the field that built the foundation of strict clinical definitions of resistance with defined breakpoints [117, 118, 119, 120].

In order to look into the effect of temperature on resistance provided by AcrAB-TolC, the following list of objectives will be met:

- 1) Confirm that temperature does affect growth rate by obtaining growth curves at 30°C and 37°C for each strain
- 2) Obtain OD-to-CFU/ mL conversion factors for both temperatures and all strains to decide on a standard inoculum size for MIC experiments
- 3) For MIC experiments, expose bacteria to different erythromycin doses for 24 hours at both temperatures, record MICs and observe any MIC shifts. Are faster growing cells more or less sensitive to erythromycin?
- 4) If MIC shifts do occur, calculate and compare AcrB expression per cell between temperatures

It should be noted that due to the focus on bacterial growth, this research will generate kinetic data only.

By achieving the above objectives, the effect of temperature on AcrAB-TolC-mediated resistance in *E. coli* can be elucidated, which will provide a general preliminary insight into the effect of temperature on antibiotic resistance. This project may provide a stepping stone into further investigation on the influence of temperature on other resistance mechanisms deployed by different



pathogens, as well as looking into the efficacy of several classes of antibiotics in cooler and warmer conditions. Together, it is hoped that this information will help guide treatment behaviours in different climates across the globe as well as in different parts of the body to be more effective whilst reducing the impact on the rate of emerging antibiotic resistance.

## Chapter 2 – Obtaining OD-to-CFU/ mL Conversion Factors and Investigating the Effect of Temperature on the Growth Profiles of *E. coli* Strains MG1655, eTB108 and AG100-A

### 2.1 Materials and Methods

#### 2.1.1 Strains

In order to investigate the effect of temperature on antibiotic resistance, particularly that of which is provided by AcrAB-TolC, derivatives of the natural host of the efflux pump and model organism *E. coli* strain K-12 were used (Table 2.1).

Wild-type (WT) *E. coli* MG1655 will be compared to experimental strain eTB108 ('evolved TB108'), the latter which expresses a modified AcrAB-TolC where the *acrB* gene is physically-fused to superfolder green fluorescent protein gene (sfGFP) downstream [68, 121]. When expressed, fluorescence is emitted under light of a wavelength of 485nm, allowing the measurement of AcrAB-TolC abundance through fluorescence intensity (FI). Its predecessor strain TB108 was provided by Dr Tobias Bergmiller at the University of Exeter, however due to subnormal AcrAB-TolC efflux quantified by a lower MIC than another wild-type *E. coli* strain AG100 (also another K-12 derivative), TB108 was evolved by Dr Carlos Reding-Roman in order to regain wild-type efflux functionality. The MIC of eTB108 was shown to be indistinguishable from the MIC of AG100 [68].

In order to confirm the role of AcrAB-TolC in antibiotic resistance, the *acrAB* knockout strain AG100-A will be used for comparison.

**Table 2.1:** *Escherichia coli* K-12 derivative strains used in this study.

Strain	Genotype	Reference
<i>E. coli</i> AG100-A	<i>argE3 thi-3 rpsL xyl mtl supE44 ΔacrAB::Tn903</i>	122
<i>E. coli</i> MG1655	<i>ilvG-rfb-50 rph-1</i>	122
<i>E. coli</i> eTB108	MG1655 <i>acrB-sfGFP-FRT</i>	68

### 2.1.2 Media

All media listed were created using deionised water up to a volume of 1 litre, where the final mixture was sterilised by autoclaving for 20 minutes at 121°C.

**Table 2.2:** Recipes for all media used throughout study.

Media (1L)	Component(s)	Supplier
LB Agar	25g LB Powder 12g Agar Powder	Formedium Sigma Aldrich
M9 salts (20 mL component A + 20 mL component B)	<i>Component A (1L):</i> 350g K <sub>2</sub> HPO <sub>4</sub> 100g KH <sub>2</sub> PO <sub>4</sub>  <i>Component B (1L):</i> 29.4g Trisodium Citrate 50g (NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub> 10.45g MgSO <sub>4</sub>	Fisher Scientific Fisher Scientific   Fisher Scientific Fisher Scientific Fisher Scientific

When required, M9 (minimal) media was prepared fresh by supplementing M9 salts (Table 2.2) with 0.2% (weight/volume) glucose and 0.1% (w/v) casamino acids. Stock solutions of glucose and casamino acids were created using M9 salts as the liquid component (Table 2.3), where the final solution was filter-sterilised using minisart 0.2 µm single-use syringe filter (Sartorius).

**Table 2.3:** Stock solutions used to create M9 media.

Stock Solution (200mL)	Components	Supplier
20% Glucose	40g Glucose	Sigma Aldrich
10% Casamino acids	20g casamino acids	Formedium

### 2.1.3 Growth Curve Protocol

In order to investigate the effect of temperature on the growth of the strains of *E. coli*, a pure culture for each strain was created by streak-planting a loopful of frozen stock onto LB agar. The plate was then placed into a 30°C incubator for 18 hours overnight. After, a single colony was picked and inoculated into 25 mL of M9 media, where the resultant mixture was placed in a shaking incubator at 30°C at 180 rpm for 18 hours.

The overnight culture was then successively diluted 1:1000 in M9 media in 3 different conical flasks (for a biological triplicate), after which the optical density (OD) was immediately read and recorded 3 times per flask (for a technical triplicate) using a digital colorimeter (Fisher Scientific) (Fig. 2.1A). Thereafter, the flasks were placed in a shaking incubator at 180 rpm set at the experimental temperature of either 30°C or 37°C, where the OD was read in the aforementioned manner hourly up to 31 hours.

### 2.1.4 Obtaining OD-to-CFU/ mL Conversion Equations

Simultaneously, the colony forming unit per mL (CFU/ mL) for the 6<sup>th</sup>, 8<sup>th</sup>, 24<sup>th</sup>, 26<sup>th</sup>, 28<sup>th</sup> and 30<sup>th</sup> hour was measured to obtain an average OD-to-CFU conversion equation for each strain at each temperature. This would enable a standard inoculum size to be used for subsequent experiments. After recording the OD at the hours mentioned above, a small sample was taken from each culture flask for a 2-fold serial dilution, resulting in a total of 5 dilutions plus the original undiluted sample. A microplate was used to hold the large number of dilutions for all three biological samples (Fig. 2.1B).

After the OD was read for each 2-fold dilution, the next step was to create a series of further 10-fold dilutions on the same microplate, of which the number of viable cells per mL would be countable when a 10 µL drop was plated onto LB agar i.e. between 30 to 300 colonies. The dilutions were 10<sup>-2</sup>, 10<sup>-4</sup>, 10<sup>-5</sup>, 10<sup>-6</sup> and 10<sup>-7</sup>, but only four were drop-plated at a time depending on the turbidity of the culture i.e. for the 6<sup>th</sup> and 8<sup>th</sup> hour, dilutions 10<sup>-2</sup>, 10<sup>-4</sup>, 10<sup>-5</sup> and 10<sup>-6</sup> were plated, whereas for the 24<sup>th</sup>, 26<sup>th</sup>, 28<sup>th</sup> and 30<sup>th</sup> hour, 10<sup>-4</sup>, 10<sup>-5</sup>, 10<sup>-6</sup> and 10<sup>-7</sup> were plated due to a higher bacterial density as a result of longer growth time.

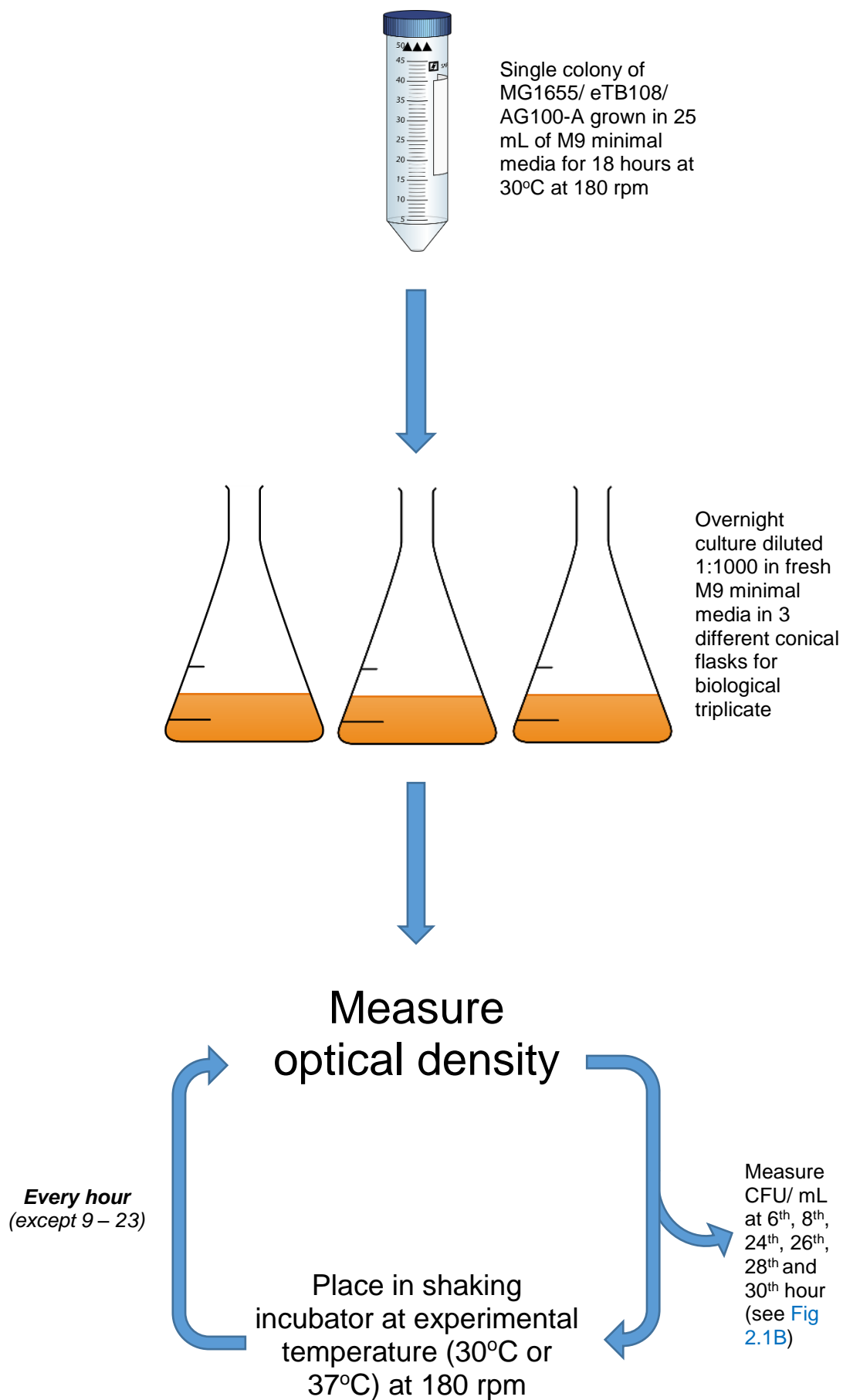
Each LB plate used was split in half, where one side contained three 10  $\mu$ L drops (technical triplicate) of one dilution e.g.  $10^{-2}$  and the other side another dilution e.g.  $10^{-4}$ .

Once dried, the plates were then placed in an incubator overnight at 30°C to allow colonies to grow. One dilution with countable colonies per 2-fold dilution (including the undiluted sample) was then picked and enumerated, and the CFU/ mL calculated using the following equation:

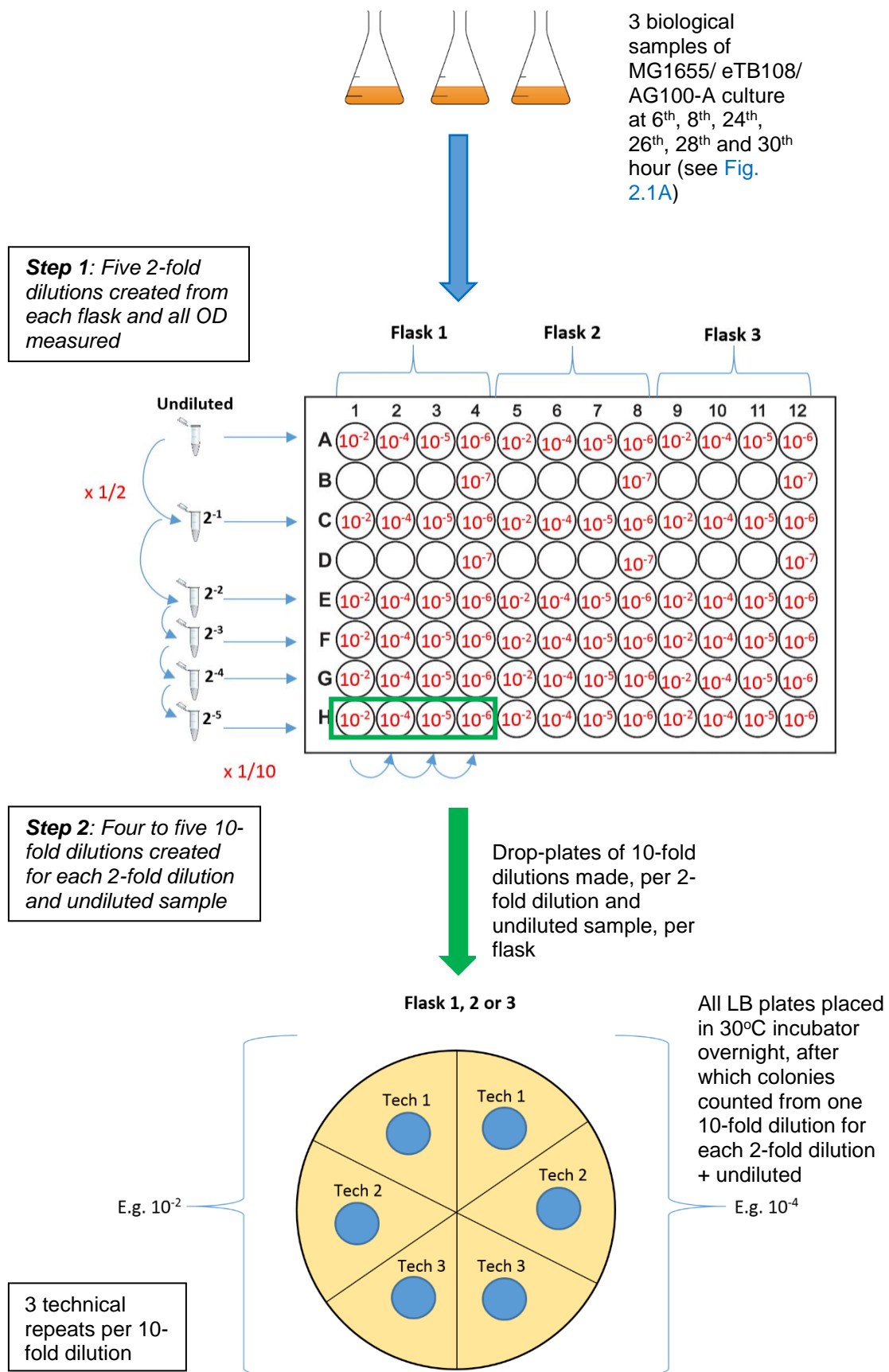
$$\text{CFU/ mL} = \frac{\text{number of colonies} \times \text{dilution factor}}{\text{volume of culture plated}}$$

The CFU/ mL was then plotted against the recorded OD for all 2-fold dilutions and undiluted samples to obtain a regression of that time point, and the equation of the line averaged out over all 6 time points to obtain one equation. As mentioned previously, this mean function will act as the OD-to-CFU/ mL conversion equation for that strain and particular temperature, as it averages out potential variations in the relationship between OD and CFU/ mL that may occur over the whole growth cycle of the *E. coli* strain e.g. changes in cell size [123], where larger cells may give the same CFU/ mL measurement but a higher OD read.

Please note that due to the measurements being manual, the OD was not read nor the culture plated for colony counts between the hours of 9 and 23, during which the culture was left overnight in the shaking incubator at the settings previously specified.



**Figure 2.1A:** A schematic of the general growth curve protocol used in this study for each strain and experimental temperature.

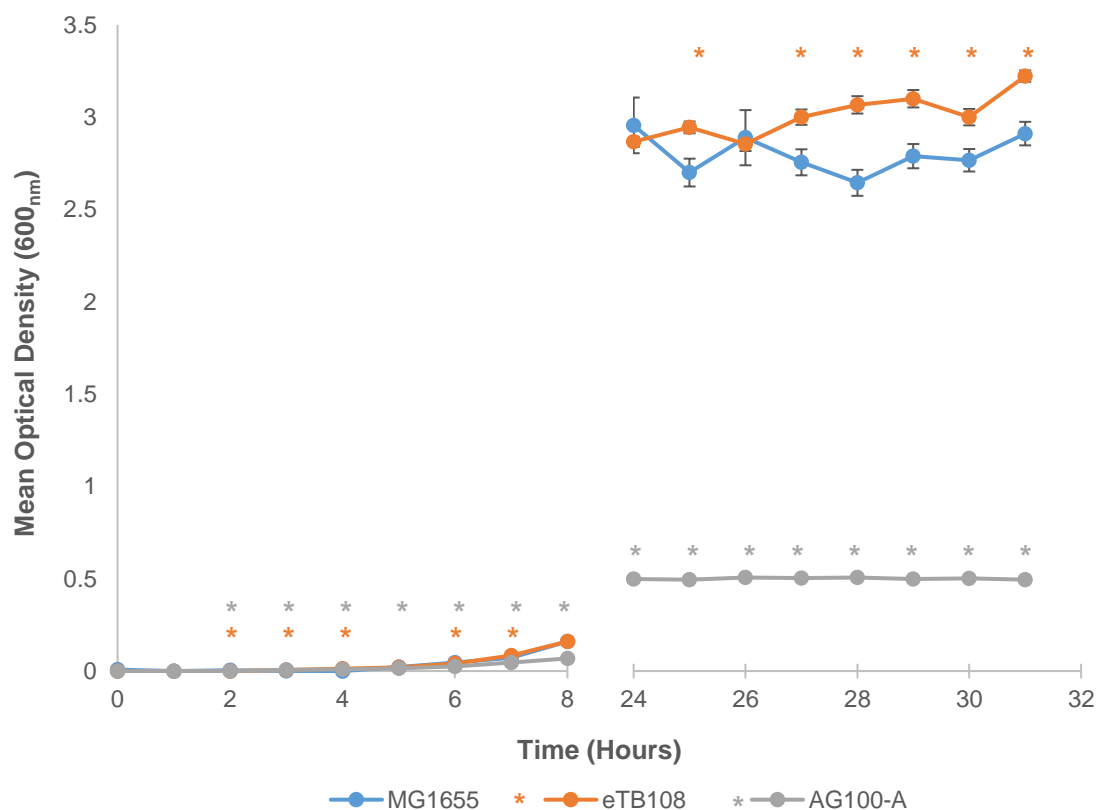


**Figure 2.1B:** A schematic of the protocol used to measure OD and CFU/ mL of a series of 2-fold dilutions for the 6<sup>th</sup>, 8<sup>th</sup>, 24<sup>th</sup>, 26<sup>th</sup>, 28<sup>th</sup> and 30<sup>th</sup> hour of culture growth. The green rectangle represents an example set of dilutions that may be plated for CFU counting. OD vs CFU/ mL graph will enable deduction of an OD-to-CFU/ mL conversion equation for each strain and temperature.

## 2.2 Results

### 2.2.1 A Comparison of Growth Curves between Strains

The OD data obtained from the growth curve protocol for each strain were plotted against time and compared.

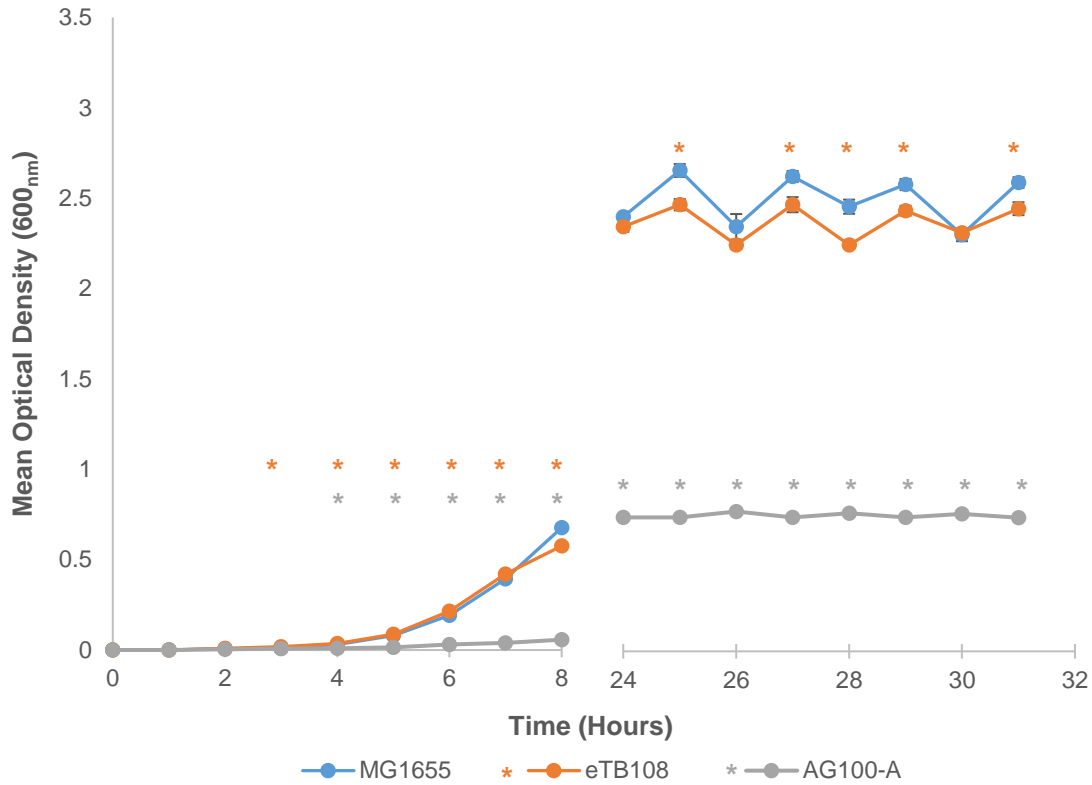


**Figure 2.2:** Growth curves over time for *E. coli* strains MG1655 (WT, blue), eTB108 (*acrB-sfGFP*, orange) and AG100-A ( $\Delta$ *acrAB*, grey) at 30°C. Overnight culture of each strain diluted 1:1000 in M9 media and allowed to incubate up to 31 hours. OD read hourly (except between 9 - 23 hours). Data points include biological and technical triplicate, where error bars represent standard error of the mean. Error bars may be small and hidden behind data points. Asterisk (\*) placed above data points indicates a statistically-significant difference of eTB108 (orange) or AG100-A (grey) from the WT growth profile, where  $p < 0.05$  using an unpaired t-test (see [Table S1](#) in supplementary for exact  $p$ -values).

At 30°C ([Fig. 2.2](#)), WT strain MG1655's lag phase occurs between hours 0 – 4, progresses through to exponential phase where it eventually approaches a potential stationary phase at (or possibly before) 24 hours, reaching an average carrying capacity of  $2.8 \pm 0.036$  OD. It can also be seen that eTB108 follows a similar pattern over time, however using an unpaired t-test confirmed that the mean OD reads for eTB108 was statistically higher than those of MG1655 ( $p < 0.05$ ) throughout most of the time points. However, statistical differences do not occur completely throughout the latter half of the growth curve, since at the 24<sup>th</sup>



and 26<sup>th</sup> hour, the mean OD of MG1655 and eTB108 are statistically similar. Nonetheless, eTB108 reaches an average carrying capacity of  $3 \pm 0.041$  OD, which is statistically higher than that of MG1655 ( $p = 0.0019$ ). The *acrAB*-knockout strain AG100-A grew to a significantly lower carrying capacity in contrast to the other two strains. In fact, the final average OD measured was at least 5-fold less than that of the others ( $0.5 \pm 0.00031$  OD).

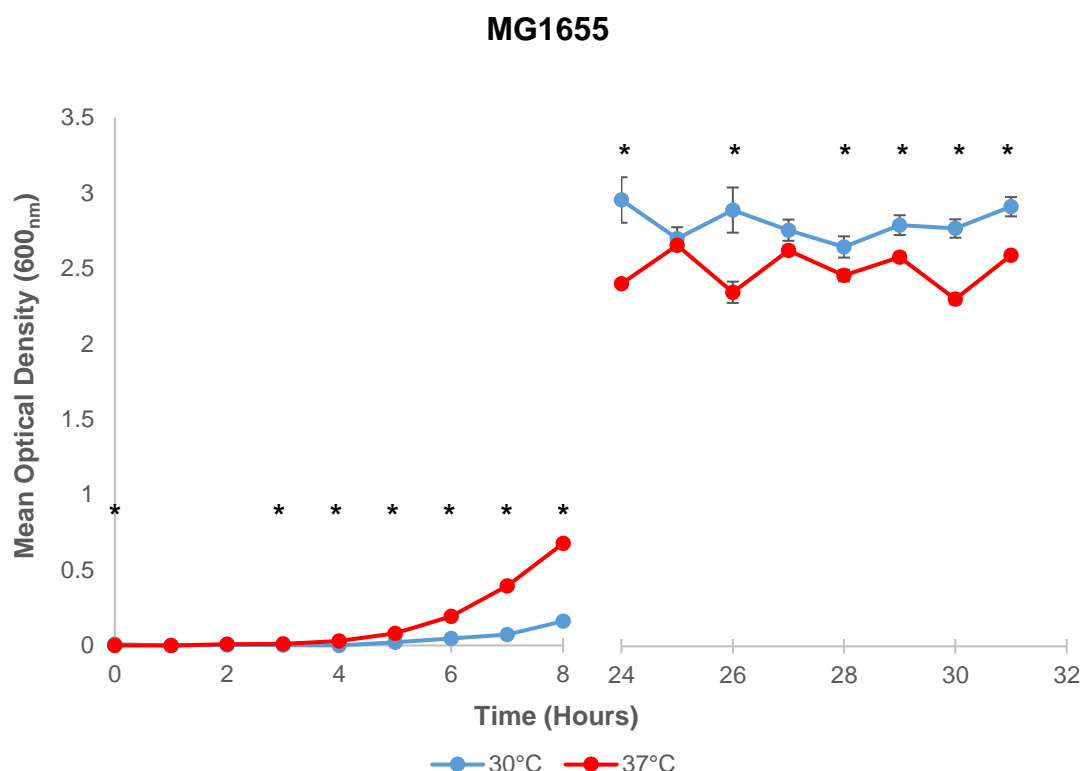


**Figure 2.3:** Growth curves over time for *E. coli* strains MG1655 (WT, blue), eTB108 (*acrB-sfGFP*, orange) and AG100-A ( $\Delta$ *acrAB*, grey) at 37°C. Overnight culture of each strain diluted 1:1000 in M9 media and allowed to incubate up to 31 hours. OD read hourly (except between 9 - 23 hours). Data points include biological and technical triplicate, where error bars represent standard error of the mean. Error bars may be small and hidden behind data points. Asterisk (\*) placed above data points indicates a statistically-significant difference of eTB108 (orange) or AG100-A (grey) from the WT growth profile, where  $p < 0.05$  using an unpaired t-test (see Table S2 in supplementary for exact  $p$ -values).

At 37°C (Fig. 2.3), similar to the results obtained at 30°C (Fig. 2.2), some OD measurements in the latter half of growth are significantly different between MG1655 and eTB108 (25<sup>th</sup>, 27<sup>th</sup>, 28<sup>th</sup>, 29<sup>th</sup> and 31<sup>st</sup> hour), whilst others are not (24<sup>th</sup>, 26<sup>th</sup> and 30<sup>th</sup> hour). However, unlike 30°C, MG1655 reaches a statistically higher average carrying capacity of  $2.49 \pm 0.045$  OD ( $p = 0.0409$ ), whilst eTB108 plateaus at  $2.37 \pm 0.032$  OD. Furthermore, the mean carrying capacity of AG100-A is again significantly lower than that of MG1655, at approximately  $0.75 \pm 0.005$  OD ( $p < 0.0001$ ).

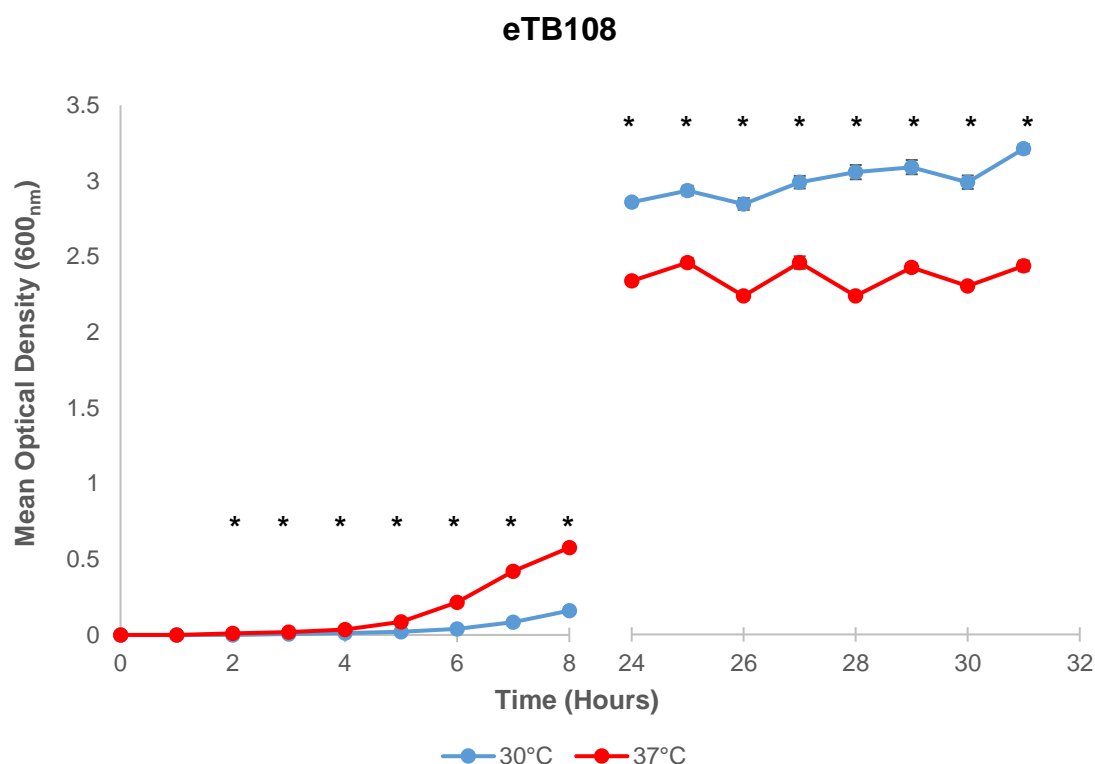
## 2.2.2 A Comparison of Growth Curves between Temperatures

The OD data was then plotted separately for each strain to see how an increase in temperature affected their growth.



**Figure 2.4:** Growth curves over time for *E. coli* strain MG1655 (WT) at 30°C (blue) and 37°C (red). Overnight culture of strain diluted 1:1000 in M9 media and allowed to incubate up to 31 hours. OD read hourly (except between 9 - 23 hours). Data points include biological and technical triplicate, where error bars represent standard error of the mean. Error bars may be small and hidden behind data points. Asterisk (\*) placed above data points indicates a statistically-significant difference in OD between temperatures, where  $p < 0.05$  using an unpaired t-test (see [Table S3](#) in supplementary for exact  $p$ -values).

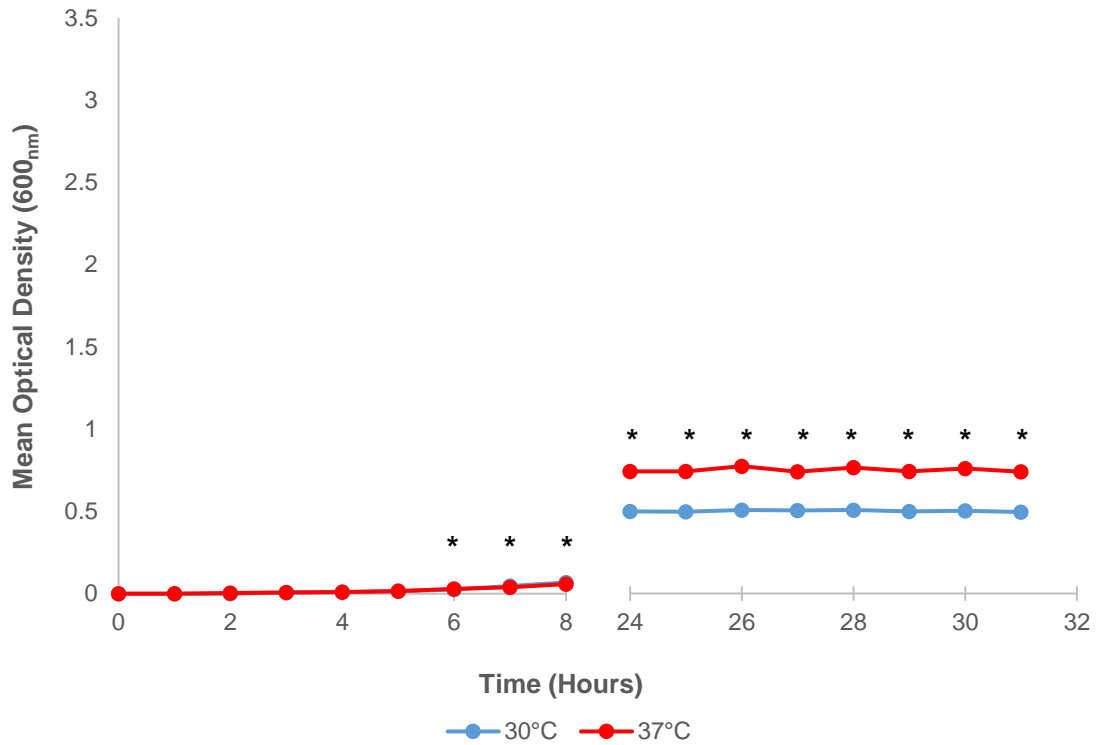
When the temperature is increased from 30°C to 37°C, the earliest part of division i.e. 3 to 8 hours is observed to be significantly quicker for MG1655 ([Fig. 2.4](#)). This may suggest that the cells are dividing much more quickly at 37°C, however more data points will be required to calculate and compare growth rates since with the current data the exponential phase cannot be visualised. In addition, the overall OD recorded at the potential stationary phase (24 – 31 hours) is statistically higher at 30°C ( $2.8 \pm 0.036$  OD) than at 37°C ( $2.49 \pm 0.045$  OD), where  $p < 0.0001$ .



**Figure 2.5:** Growth curves over time for *E. coli* strain eTB108 (*acrB-sfGFP*) at 30°C (blue) and 37°C (red). Overnight culture of strain diluted 1:1000 in M9 media and allowed to incubate up to 31 hours. OD read hourly (except between 9 - 23 hours). Data points include biological and technical triplicate, where error bars represent standard error of the mean. Error bars may be small and hidden behind data points. Asterisk (\*) placed above data points indicates a statistically-significant difference in OD between temperatures, where  $p < 0.05$  using an unpaired t-test (see [Table S4](#) in supplementary for exact  $p$ -values).

With regards to the potential difference in growth rates, the same applies to eTB108 ([Fig. 2.5](#)) where it may be significantly higher in 37°C but difficult to firmly determine without further data points. The carrying capacities on the other hand are again statistically different ( $p < 0.0001$ ), where the average final OD obtained at 30°C is significantly higher ( $3 \pm 0.041$  OD) than that obtained at 37°C ( $2.37 \pm 0.032$  OD).

## AG100-A



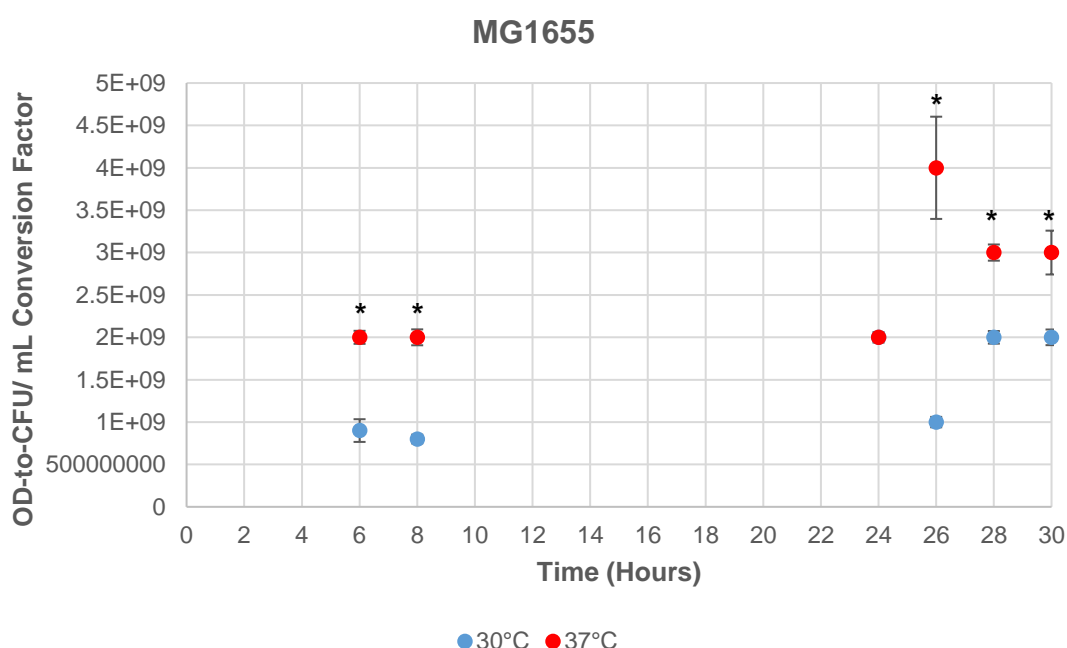
**Figure 2.6:** Growth curves over time for *E. coli* strain AG100-A ( $\Delta acrAB$ ) at 30°C (blue) and 37°C (red). Overnight culture of strain diluted 1:1000 in M9 media and allowed to incubate up to 31 hours. OD read hourly (except between 9 - 23 hours). Data points include biological and technical triplicate, where error bars represent standard error of the mean. Error bars may be small and hidden behind data points. Asterisk (\*) placed above data points indicates a statistically-significant difference in OD between temperatures, where  $p < 0.05$  using an unpaired t-test (see [Table S5](#) in supplementary for exact  $p$ -values).

In contrast, from the OD data obtained for AG100-A at both temperatures ([Fig 2.6](#)), the mean carrying capacity is significantly higher at 37°C ( $0.75 \pm 0.005$  OD) than at 30°C ( $0.5 \pm 0.0016$  OD), where  $p < 0.0001$ .

## 2.2.3 OD-to-CFU/ mL Conversion

### 2.2.3.1 Conversion Factor per Time Point

As mentioned previously in section 2.1.4, for the hours 6, 8, 24, 26, 28 and 30, OD was plotted against CFU/ mL to obtain regressions in the form of  $\text{CFU/ mL} = \text{OD} \times \text{CF}$ , where CF represents the OD-to-CFU/ mL conversion factor (see [Fig. S1 - S6](#) and [Table S10](#) in supplementary). The conversion factors were then plotted over time for each strain and temperature below. Note that large numbers on the y-axis are in exponential notation ( $E + n$ ), where E multiplies the previous number by 10 to the  $n$ th power e.g.  $5E+09$  represents  $5 \times 10^9$ .

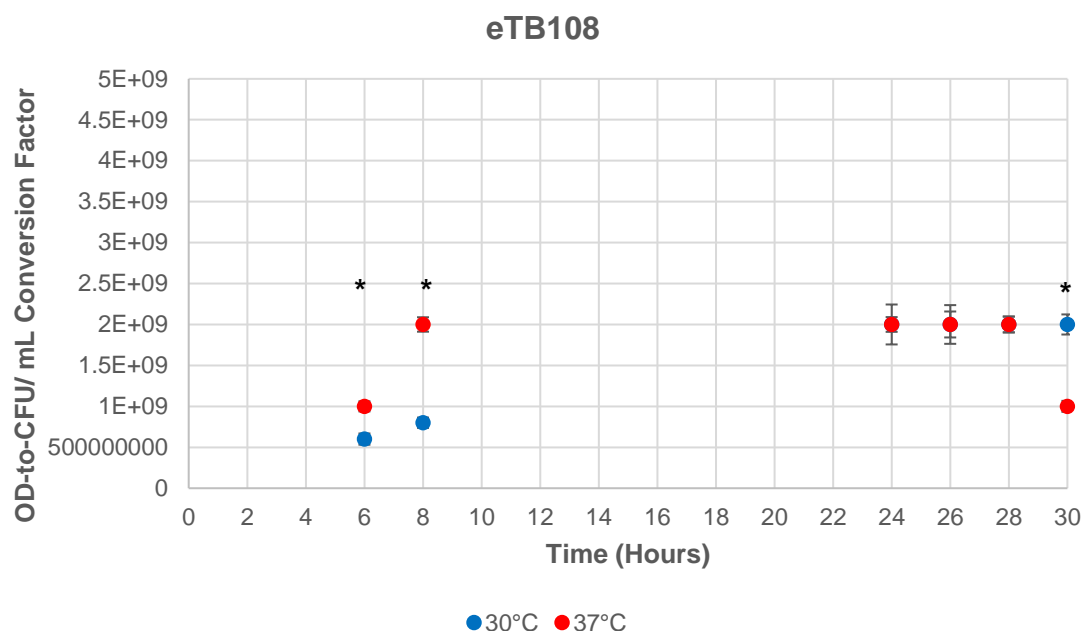


**Figure 2.7:** OD-to-CFU/ mL conversion factor over time for *E. coli* strain MG1655 (WT) at 30°C (blue) and 37°C (red). Overnight culture of strain diluted 1:1000 in M9 media and allowed to incubate up to 31 hours. For hours 6, 8, 24, 26, 28 and 30, OD and CFU/ mL recorded for half dilutions to obtain equation of line i.e. OD-to-CFU/ mL conversion factors. Data points include biological and technical triplicate, where error bars represent standard deviation of the regression slope. Error bars may be small and hidden behind data points. Asterisk (\*) placed above data points indicates a statistically-significant difference between temperatures, where  $p < 0.05$  using an unpaired t-test (see [Table S6](#) in supplementary for exact  $p$ -values).

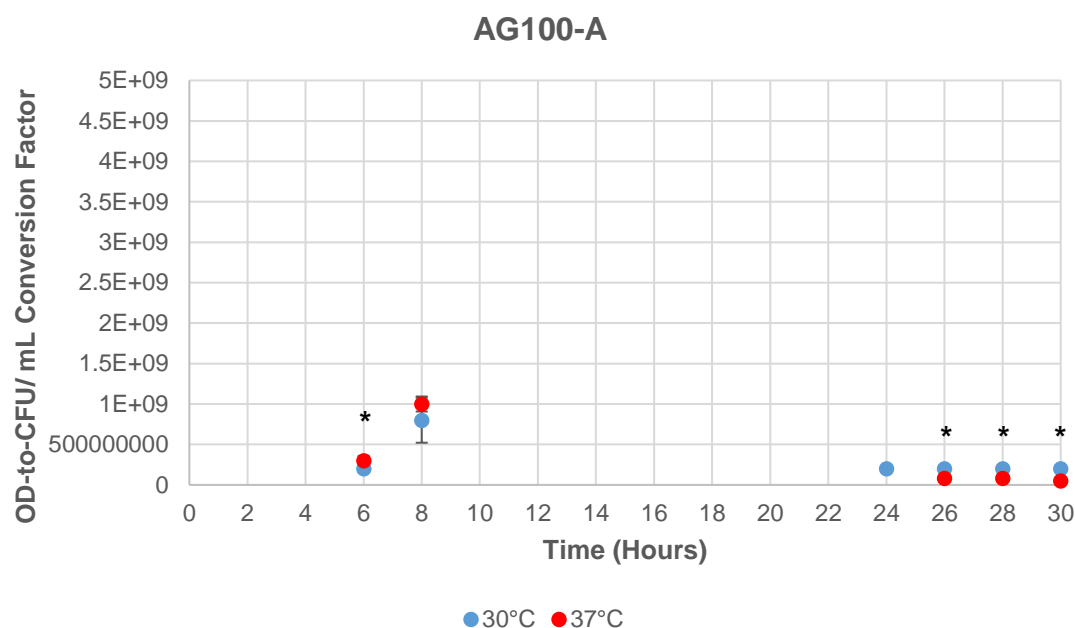
For MG1655 ([Fig. 2.7](#)), at most of the time points (with the exception of the 24<sup>th</sup> hour), there is a statistically significant difference in the OD-to-CFU/ mL conversion factor between the two temperatures, where there are more viable cells per OD in 37°C.

Trends are not nearly as evident in eTB108 (Fig. 2.8) and AG100-A (Fig. 2.9), where between the temperatures the OD-to-CFU/ mL conversion factor is either statistically the same (for eTB108, hours 24, 26, 28 and AG100-A, hours 6 and 8) significantly higher in 30°C and less in 37°C (for eTB108, the 30<sup>th</sup> hour and AG100-A, hours 26, 28 and 30) or vice versa (eTB108 hours 6 and 8).

Nonetheless, for better comparison between strain and temperature as well as deciding on an appropriate inoculum size for the next set of experiments, the values for all time points were averaged out and plotted to give a mean conversion factor for each experimental condition (Fig. 2.10).



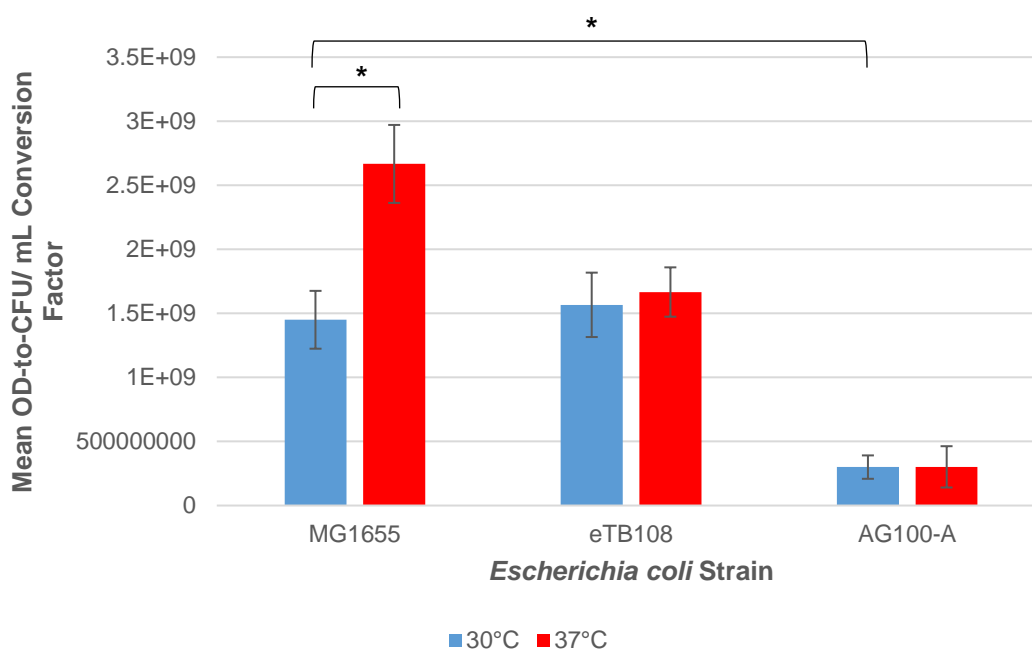
**Figure 2.8:** OD-to-CFU/ mL conversion factor over time for *E. coli* strain eTB108 (*acrB-sfGFP*) at 30°C (blue) and 37°C (red). Overnight culture of strain diluted 1:1000 in M9 media and allowed to incubate up to 31 hours. For hours 6, 8, 24, 26, 28 and 30, OD and CFU/ mL recorded for half dilutions to obtain equation of line i.e. OD-to-CFU/ mL conversion factors. Data points include biological and technical triplicate, where error bars represent standard deviation of the regression slope. Error bars may be small and hidden behind data points. Asterisk (\*) placed above data points indicates a statistically-significant difference between temperatures, where  $p < 0.05$  using an unpaired t-test (see Table S7 in supplementary for exact  $p$ -values).



**Figure 2.9:** OD-to-CFU/ mL conversion factor over time for *E. coli* strain AG100-A ( $\Delta acrAB$ ) at 30°C (blue) and 37°C (red). Overnight culture of strain diluted 1:1000 in M9 media and allowed to incubate up to 31 hours. For hours 6, 8, 26, 28 and 30, OD and CFU/ mL recorded for half dilutions to obtain equation of line i.e. OD-to-CFU/ mL conversion factors. Observations at 24 hours at 37°C have been omitted due to error. Data points include biological and technical triplicate, where error bars represent standard deviation of the regression slope. Error bars may be small and hidden behind data points. Asterisk (\*) placed above data points indicates a statistically-significant difference between temperatures, where  $p < 0.05$  using an unpaired t-test (see [Table S8](#) in supplementary for exact  $p$ -values).

### 2.2.3.2 Mean OD-to-CFU/ mL Conversion Factors and Standardising the Inoculum Size

Apart from MG1655, the average OD-to-CFU/ mL conversion factor does not change with increasing temperature for the other strains (Fig. 2.10). There is approximately a 1.8-fold increase for MG1655 when increasing the temperature to 37°C. It should also be noted that, relative to the mean value reported at 30°C for MG1655, both values for eTB108 are statistically similar, whereas both conversion rates for AG100-A are ~5-fold less. The mean values in Table 2.4 were used to standardise the inoculum size for subsequent experiments and to calculate AcrB expression per cell in eTB108 (section 3.2.4).



**Figure 2.10:** Average OD-to-CFU/ mL conversion factor for *E. coli* strains MG1655 (WT), eTB108 (*acrB-sfGFP*) and AG100-A ( $\Delta$ *acrAB*) at 30°C (blue) and 37°C (red). Overnight culture of strain diluted 1:1000 in M9 media and allowed to incubate up to 31 hours. For hours 6, 8, 24, 26, 28 and 30, OD and CFU/ mL recorded for half dilutions to obtain equation of line i.e. OD-to-CFU/ mL conversion factors. Factors averaged out over time points to give mean conversion factor per temperature per strain. Data points include biological and technical triplicate, where error bars represent standard error of the mean. Statistical significance between two values where  $p < 0.05$  using an unpaired t-test is represented by an asterisk (\*) (see Table S9 in supplementary for exact  $p$ -values).



**Table 2.4:** Mean OD-to-CFU/ mL conversion factor per strain and temperature.

<i>E. coli</i> Strain	OD-CFU/ mL Conversion Factor	
	30°C Mean ± SEM (x 10 <sup>8</sup> )	37°C Mean ± SEM (x 10 <sup>8</sup> )
<b>MG1655 (WT)</b>	<b>14.5 ± 2.3</b>	<b>26.7 ± 3</b>
<b>eTB108 (<i>acrB-sfGFP</i>)</b>	<b>15.7 ± 2.5</b>	<b>16.7 ± 1.9</b>
<b>AG100-A (<math>\Delta</math><i>acrAB</i>)</b>	<b>3 ± 0.9</b>	<b>2.7 ± 1.6</b>

Note: see [Fig. S1 – S6](#) and [Table S10](#) in supplementary for individual conversion factors per time point, prior to averaging, and their corresponding  $R^2$  values

For simplicity, the chosen inoculum size for the next set of experiments in chapter 3 was  $2.03 \times 10^9$  cells per mL, as this is the same number that MG1655 reaches when grown at 30°C for approximately 18 hours of culture time (~1.4 OD). Therefore, this will require the overnight culture for the experiments to reach an OD of 1.3 for eTB108 and 6.8 for AG100-A when also grown at 30°C.

## 2.3 Discussion

The primary aim of this chapter was to obtain growth curves, CFUs during growth, and ultimately OD-to-CFU/ mL conversion factors for *E. coli* strains MG1655, eTB108 and AG100-A at both 30°C and 37°C. This would help standardise the inoculum size for the next set of experiments in chapter 3, and was achieved by allowing each strain to grow in M9 media up to 31 hours, during which their OD was recorded and intermittently their CFU/ mL also.

During these calibration experiments, the aim was also to confirm the assumption of similarities (and if not, highlight the differences) between WT strain MG1655 and sfGFP-tagged eTB108, since the presence of sfGFP in the genome of eTB108 has been shown to not affect the efflux function of AcrAB-TolC [68].

With regards to the growth curves obtained at 30°C and 37°C (Fig. 2.2 and Fig 2.3 respectively), surprisingly eTB108 and MG1655 overall differ from one another statistically between 24 – 31 hours. In particular, at 30°C, eTB108 has a significantly higher average carrying capacity than MG1655. Initially, it could be assumed that the presence of sfGFP has somehow affected metabolism of the cell, however, if anything, the likelihood is that the presence of sfGFP may have an energy cost associated with it, albeit large or small [124]. This would imply that MG1655 should have a higher carrying capacity as it is able to utilise more energy for growth, but this is not the case at 30°C. Specifically for standard GFP, its presence has been shown to have no significant effect on the physiology or the growth of bacteria and other organisms including *E. coli* [125, 126]. The main difference between GFP and sfGFP is that sfGFP has improved folding efficiency providing enhanced fluorescence, as well as being more stable at elevated temperatures [127]. Furthermore, at 37°C, the opposite is seen where MG1655 has a higher average carrying capacity than sfGFP-tagged eTB108, further confirming that sfGFP is most likely not affecting growth. Another possibility as to why eTB108 has a greater carrying capacity at 30°C in comparison to MG1655 may be due to how the strain itself was developed. As mentioned in section 2.1.1, TB108 was evolved to regain wild-type efflux activity, which led to eTB108. Specifically, this was done by growing TB108 at 30°C via batch culture transfer into new media per day, over a total of

7 days. The bacteria were also exposed to a low basal concentration of erythromycin in order to select for populations with comparatively higher levels of efflux function [68]. During this process however, the strain may have evolved adaptive mutations that increase its fitness at 30°C, allowing more efficient growth at this temperature and thus leading to higher yield. To test this, an *acrAB* knockout strain, such as AG100-A, may be grown in the same conditions as the aforementioned evolutionary experiment. The presence of adaptive mutations may be indicated by the increase in fitness over the duration of the experiment i.e. an overall increase in carrying capacity.

In the case of the growth curves of AG100-A obtained at both temperatures (Fig 2.2, Fig 2.3 and Fig. 2.6), bacterial proliferation is considerably more stunted leading to relatively much lower carrying capacities. This may be expected, since AcrAB is postulated to be involved in the efflux of metabolic waste from processes such as enterobactin, cysteine and purine biosynthesis as well as gluconeogenesis [128]. The up-regulation of AcrAB expression has been shown to occur from the increased expression of *acrAB* operon regulators SoxS and MarA as a result of metabolite accumulation [128, 129]. Therefore the absence of AcrAB in AG100-A is likely to have led to the accumulation of metabolites within the bacterial cells, leading to potential DNA damage and protein denaturation [129, 130]. Due to the toxic effect of poor pump function, efflux pump inhibitors are being looked into as potential therapeutic agents in the clinic [131, 132, 133].

In addition, AG100-A harbours the mutation *argE3*, preventing arginine biosynthesis. Arginine is required for the synthesis of proteins and nitrogen-containing compounds in *E. coli* and other bacteria, and therefore its absence is likely to affect cell growth [134, 135, 136]. This, in combination with impaired efflux activity, are likely contributing to the lower yield observed in the above experiments.

The next step was to do a side-by-side comparison to see the effect of temperature on the growth profiles of each strain. With the exception of AG100-A (Fig. 2.6), both MG1655 (Fig. 2.4) and eTB108 (Fig 2.5) show a higher average carrying capacity at 30°C than at 37°C. This could potentially be due to a growth rate-yield trade-off i.e. the incompatibility of high biomass generation

per time with high biomass generated per mole of glucose, postulated to be as a result of thermodynamic constraints [137]. In other words, slow-growing bacterial cells are, overall, able to produce more offspring from one mole of glucose (or any other carbon source) than their faster-growing counterparts. This phenomenon is theorised to occur in natural environments as a result of competition for resources [137]. A growth rate-yield trade-off has been demonstrated empirically in *E. coli*, where the growth rate was manipulated by adapting the bacteria to the environment through serial passage [138]. Although higher cell numbers can be seen in Fig. 2.4 and Fig. 2.5 at 30°C in comparison to 37°C, coupled with the knowledge that *E. coli* cells grow faster (and at their optimal) at 37°C [100], a growth rate-yield trade-off (and a lack thereof in AG100-A, Fig 2.6) can only be speculated due to no calculated growth rates.

Growth rates could not be obtained since the exponential phase could not be visualised as a result of the absence of OD data between 9 – 23 hours. However, using an automated plate reader by which OD will be read every 20 minutes for a span of 24 hours, an attempt at investigating the effect of temperature on growth rate will be made in the next chapter.

It should be noted that slower-growing cells (specifically where their proliferation rate has been manipulated by nutrient levels) have been shown to be more tolerant to changing conditions such as sudden antibiotic exposure [102, 103, 105]. By obtaining growth rates, the next chapter will also attempt to elucidate whether this relationship between growth rate (specifically manipulated by temperature) and antibiotic susceptibility applies to *E. coli* and its response to erythromycin.

Finally, the main aim of this chapter was accomplished by obtaining the OD-to-CFU/ mL conversion factors for all three strains at 30°C and 37°C (Fig. 2.10 and Table 2.4). The relationship between OD and CFU/ mL were statistically the same for eTB108 and MG1655 at 30°C, further confirming their similarities suggesting that the expression of sfGFP, at the levels involved in this study, may not impact cell growth. Apart from eTB108 and AG100-A where the effect of temperature is negligible, MG1655 is the only strain to have a significantly higher (~2-fold increase) conversion factor at 37°C. In other words, per unit of

OD, there are almost double the number of viable cells. Why this occurs only in MG1655 and not in eTB108 may be difficult to explain without visualising the dynamics of OD and CFU/ mL over time, and any physiological changes that the bacteria may undergo as a result of temperature. It could be speculated that at 37°C, the size of bacterial cells are smaller, and assuming that they have lower energetic costs than one large cell, exist in almost twice the amount per mole of glucose in comparison to 30°C. An increase in temperature has been shown to reduce cell size in *E. coli* in past studies [139, 140]. However, again if this were the case, why is the same relationship between OD and CFU/ mL not observed in eTB108 when increasing the temperature from 30°C to 37°C? As well as comparing CFU/ mL dynamics with OD, the theory that MG1655 cells may become smaller at higher temperature can be tested via single-cell microfluidics [141], where changes in cell size can be physically observed in real-time whilst manipulating the temperature.

Nonetheless, as mentioned previously, these OD-to-CFU/ mL conversion factors will serve their purpose in the following chapter.

A limitation of this study is that the OD measurements taken by the spectrophotometer may be close or even below the detection threshold of the equipment, particularly in the earlier hours of bacterial growth. Furthermore, hourly conversion factors were derived for limited time points (6<sup>th</sup>, 8<sup>th</sup>, 24<sup>th</sup>, 26<sup>th</sup>, 28<sup>th</sup> and 30<sup>th</sup>) and averaged for comparability between strains and temperature, which may not be representative of the whole growth curve nor of the specific OD-to-CFU/ mL relationship for each time point/ growth phase. This may lead to potential underestimation/ overestimation of the CFU/ mL dependent on the total duration of cell growth. If the experiments described in sections 2.1.3 and 2.1.4 were to be repeated, a more accurate method would be to feasibly determine conversion factors at regular intervals throughout the whole growth cycle (within all growth phases), and for each strain/ temperature choosing one time point confidently within stationary phase to determine the inoculum size. This will enable the inoculum size to be more easily achieved by growing the strains for a predetermined amount of time under the standard conditions and specific temperature, with the cells at the same growth stage, minimising variability between experiments.

## **Chapter 3 – The Effect of Temperature on the MIC of MG1655, eTB108 and AG100-A, and on the Selection of Pump Expression**

The overall aim of this chapter is to investigate the effect of a temperature increase on the susceptibility of *E. coli* to erythromycin and ultimately, changes in the expression of AcrB in order to understand the selection on pump expression under thermal variation.

### **3.1 Materials and Methods**

The *E. coli* strains described in section 2.1.1 and M9 media described in section 2.1.2 will be used throughout the following experiments in this chapter.

#### **3.1.1 Erythromycin Stock**

10 mg/ mL stock solutions of erythromycin were made by dissolving 100 mg of erythromycin powder, provided by Abcam Ltd. (AB141205), into 10mL of pure ethanol and then aliquoted into 1mL eppendorf tubes. All aliquots were maintained in -20°C for no longer than 1 month as per product instructions.

#### **3.1.2 Plate Reader Setup**

To be able to see the effects of temperature on the MIC of each strain, and AcrB expression in eTB108, the FLUOstar OPTIMA plate reader was used which is capable of measuring OD and FI simultaneously.

Upon start up, the plate reader was set up to heat and maintain the experimental temperature (30°C or 37°C). A script was curated for the plate reader to follow, which involved OD and FI readings of each well every 20 minutes for a total duration of ~24 hours. Specifically, this consisted of approximately 5 minutes of shaking time at medium strength, and 15 minutes of OD and FI measurement three times per well in a 2x2 matrix. OD was read using the 600nm filter and sfGFP FI was read using the excitation/ emission wavelengths of 485/ 520nm.

### 3.1.3 MIC Assay

As the MIC can vary for different experiments based on strain, media and many other factors, this study will involve obtaining MICs specific to the experimental strains and media used in this research project.

In order to obtain an MIC for each strain and temperature, a single colony was picked from a streak plate and inoculated into 25 mL of M9 minimal media. The mixture was then placed into a shaking incubator at 30°C at 180 rpm for 18 hours. After, the overnight culture was then used to create three more overnight cultures by making three 1:1000 dilutions and incubating in a similar manner to the above for a biological triplicate.

The aim was for the second set of cultures to reach a cell number of  $2.03 \times 10^9$  per mL based on the deduced OD-to-CFU/ mL conversion factors ([Table 2.4](#)), which would require an OD of 1.4 for MG1655 and 1.3 for eTB108, easily achieved after 18 hours of incubation. However, as AG100-A required an overnight culture of 6.8 OD, a much higher OD that surpasses the carrying capacity of ~0.5 OD recorded in [Fig. 2.2](#), the second set of AG100-A overnight cultures were made to be significantly larger in volume. From these larger cultures, a sample was taken and exposed to a cycle of centrifugation and resuspension with more culture until the desired OD was achieved, a standard microbiological procedure to concentrate bacteria in a sample [[142](#), [143](#), [144](#), [145](#), [146](#)].

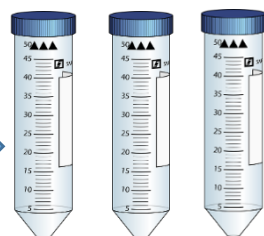
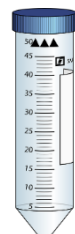
After, for each strain, the three overnight cultures (now experimental samples) were transferred onto a microplate in the manner shown in [Fig. 3.1](#), where three columns were dedicated to each sample and each well contained 150 µl of said sample. Columns 10, 11 and 12 remained empty to prevent inoculation of *E. coli* cells into the blank (explained further below). A sterile 96-pin replicator (Boeckel Scientific, US), was then used to transfer 1% of each well onto a microplate that contained wells of 150 µl of M9 media supplemented with erythromycin to create a concentration gradient ranging from 0 - 70 µg/ mL down each of the rows. This approximated to  $3.04 \times 10^6$  cells being introduced into the new wells for each strain. Due to the empty columns on the previous

plate, the final three columns of the new plate contained M9 media only without any cells as a blank. The freshly inoculated microplate was then placed into the plate reader at either 30°C or 37°C in which the OD and FI were recorded every 20 minutes up to 24 hours (in the manner stated above in section 3.1.2).

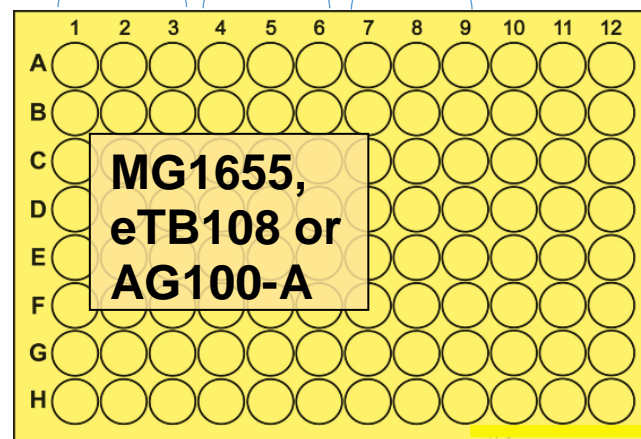


Overnight culture grown in 30°C in shaking incubator at 180 rpm for 18 hours.

1:1000<sup>th</sup> dilution of overnight culture to make 3 more cultures of required OD i.e. biological triplicate. Once again, inoculated vials placed in 30°C shaking incubator at 180 rpm overnight.

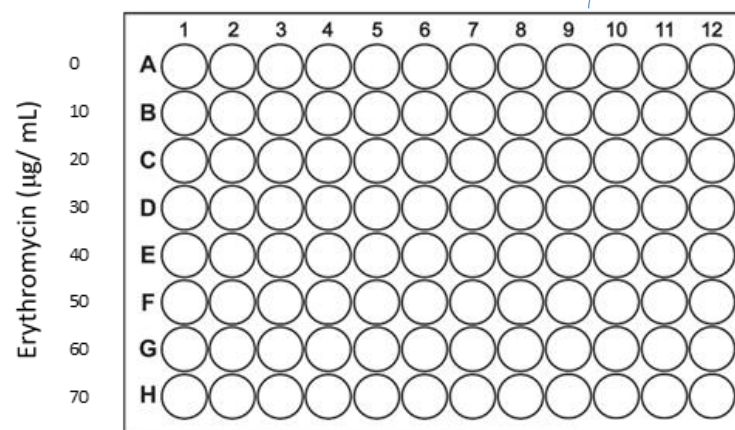


Sample 1    Sample 2    Sample 3



150 µL of sample (containing  $2.03 \times 10^9$  cells per mL) placed in each well. Column 10-12 left empty.

M9 Only



1% of each well ( $\sim 3.04 \times 10^6$  cells) inoculated into wells of new plate with erythromycin gradient and fresh M9 media. Rows 10-12 not inoculated to serve as blank.

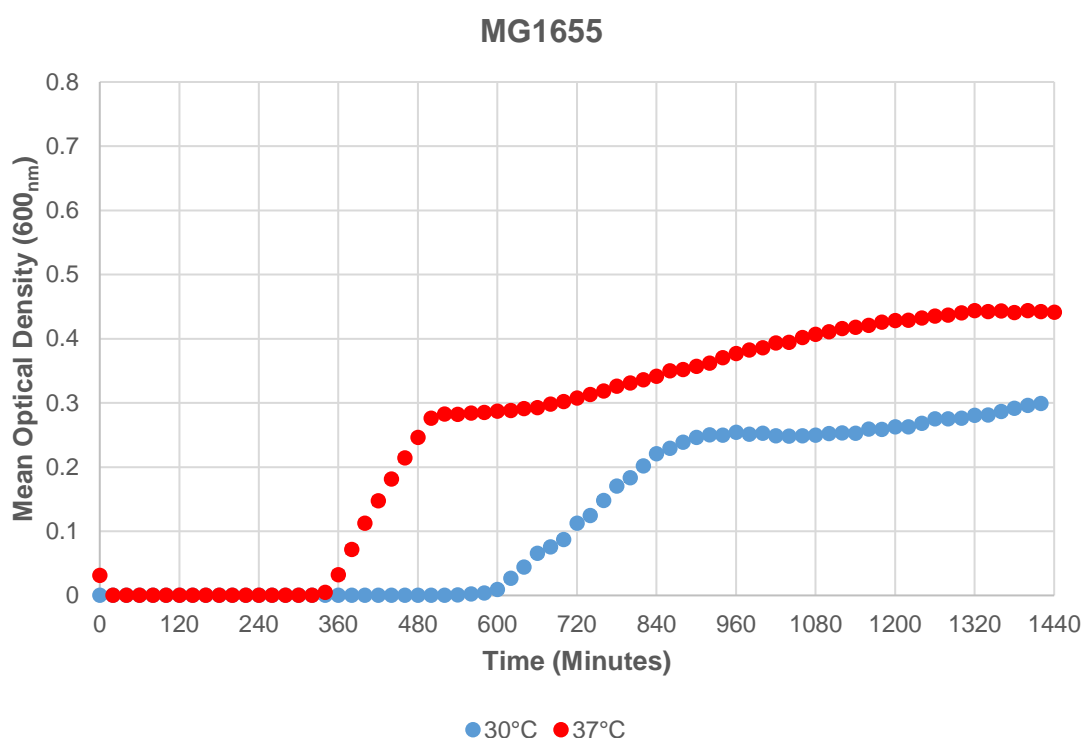
Place into plate reader at 30°C/ 37°C to read **optical density and fluorescence intensity** every 20 minutes up to 24 hours.

**Figure 3.1:** The overall protocol used to inoculate microplates with *E. coli* strains MG1655, eTB108 or AG100-A to determine MICs and measure FI using plate reader.

## 3.2 Results

Due to the intermittent reading of OD every 20 minutes over the span of 24 hours using the plate reader, the data provided by the wells that were not exposed to erythromycin (A1 – A9, [Fig. 3.1](#)) were exploited to visualise the growth cycle for each strain at 30°C and 37°C ([Fig. 3.2 – 3.4](#)).

### 3.2.1 Growth Rate Increases with Temperature

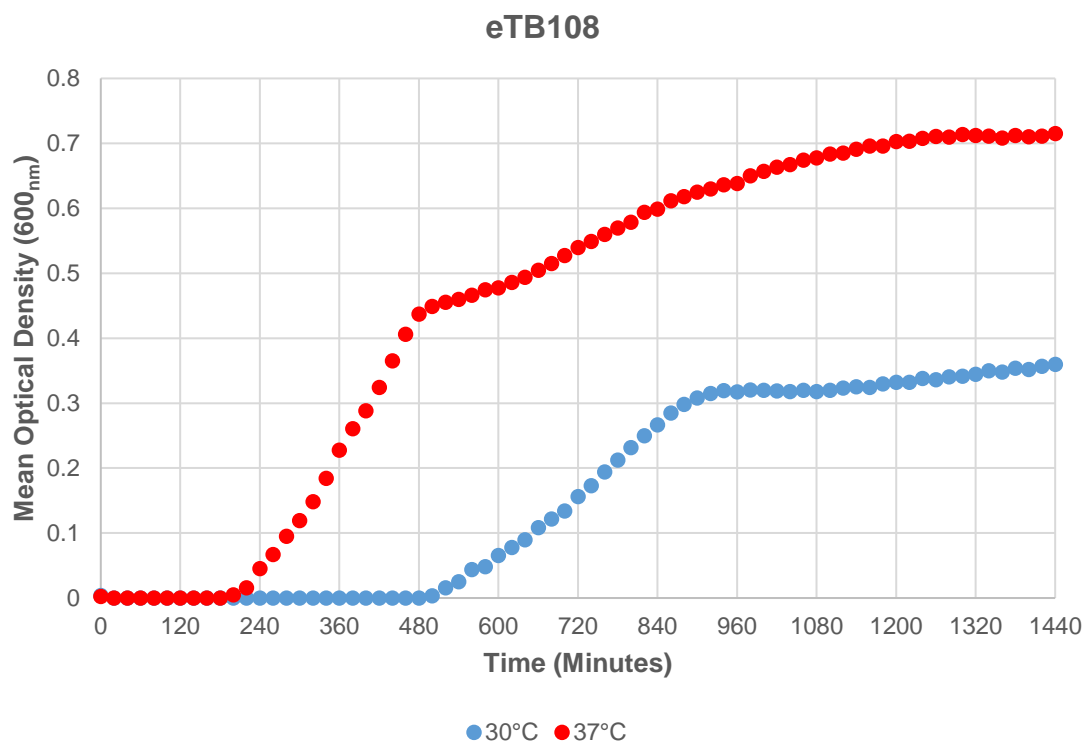


**Figure 3.2:** Growth curves over time for *E. coli* strain MG1655 (WT) at 30°C (blue) and 37°C (red) using plate reader. OD read every 20 minutes up to 1440 minutes (24 hours). Data points include biological and technical triplicate, where error bars represent standard error of the mean. Error bars may be small and hidden behind data points.

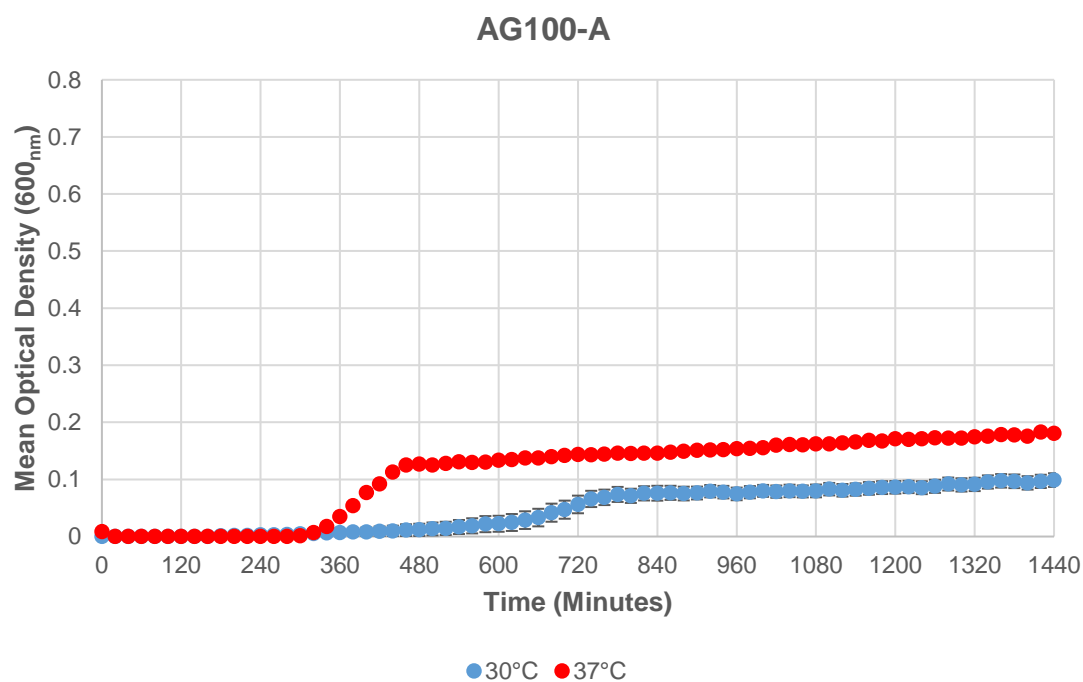
For MG1655 ([Fig. 3.2](#)), the bacteria proliferate between ~620 – 840 minutes (10.3 – 14 hours) at 30°C, and at 37°C between ~360 – 480 minutes (6 – 8 hours).

For eTB108 ([Fig. 3.3](#)), population growth occurs between ~520 – 860 minutes (8.7 – 14.3 hours) at 30°C, and at 37°C between ~240 – 460 minutes (4 – 7.7 hours).

With regards to AG100-A ([Fig. 3.4](#)), at 30°C it is much more difficult to pinpoint the time frame in which proliferation occurs due to large standard-error-of-the-mean values. At 37°C, however, the phase occurs between ~340 – 460 minutes (5.7 – 7.7 hours).



**Figure 3.3:** Growth curves over time for *E. coli* strain eTB108 (*acrB-sfGFP*) at 30°C (blue) and 37°C (red) using plate reader. OD read every 20 minutes up to 1440 minutes (24 hours). Data points include biological and technical triplicate, where error bars represent standard error of the mean. Error bars may be small and hidden behind data points.

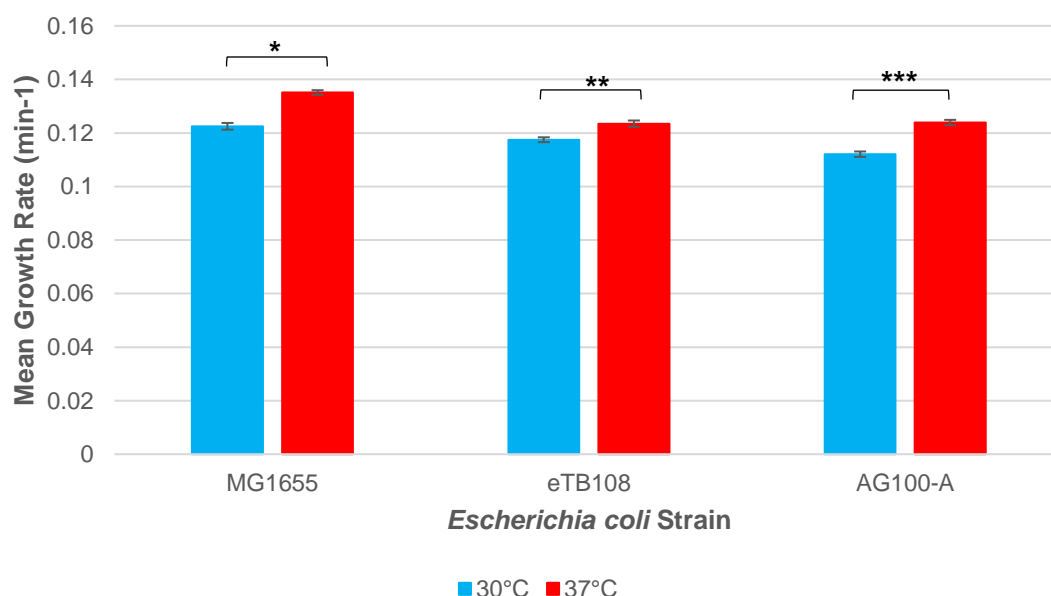


**Figure 3.4:** Growth curves over time for *E. coli* strain AG100-A ( $\Delta$ *acrAB*) at 30°C (blue) and 37°C (red) using plate reader. OD read every 20 minutes up to 1440 minutes (24 hours). Data points include biological and technical triplicate, where error bars represent standard error of the mean. Error bars may be small and hidden behind data points.

Please note that the final ODs measured by the plate reader (Fig. 3.2 – 3.4) are not the same as those measured by the colorimeter (Fig. 2.2 – 2.6), likely due to factors such as use of different machinery and thus the culture environment.

In fact, from the plate reader measurements, it can be seen that the carrying capacity is not reached for all three strains due to continuous proliferation (Fig 3.2 – 3.4). It is possible that diauxic growth is occurring, where there are visibly two cycles of growth that occur one after another [147]. Therefore potential growth rate-yield trade-offs cannot be observed with the current set of data.

Mean growth rates for all strains and temperatures were obtained from the first growth phase (within the time frames mentioned above) using the GrowthRates programme, which calculated the growth rate for wells A1 – A9 by converting the OD data into natural logarithms (ln OD) and using the range of points where the greatest slope occurs i.e. within the exponential phase [148]. All 9 growth rates from each well were then averaged and plotted (Fig. 3.5) to compare between temperatures within each strain.

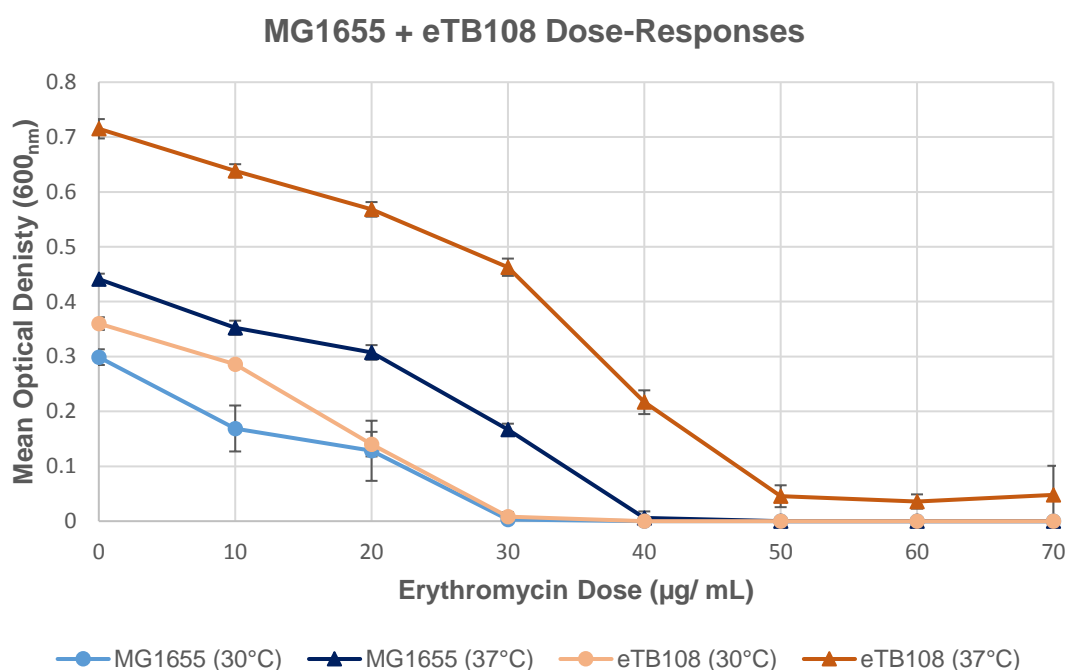


**Figure 3.5:** Mean growth rate per minute for *E. coli* strains MG1655 (WT), eTB108 (*acrB-sfGFP*) and AG100-A ( $\Delta$ *acrAB*) at 30°C (blue) and 37°C (red). Growth rates were calculated from exponential phase using GrowthRates programme [148]. Data points include biological and technical triplicate, where error bars represent standard error of the mean. Statistical significance between two values where  $p < 0.05$  using an unpaired t-test is represented by asterisk(s) (\*) (\*:  $p < 0.0001$ , \*\*:  $p = 0.001$ , \*\*\*:  $p < 0.0001$ ).

For all three strains (Fig. 3.5), it can be confirmed statistically that the growth rate is significantly higher at 37°C than at 30°C i.e. the *E. coli* strains grow more quickly at the higher temperature.

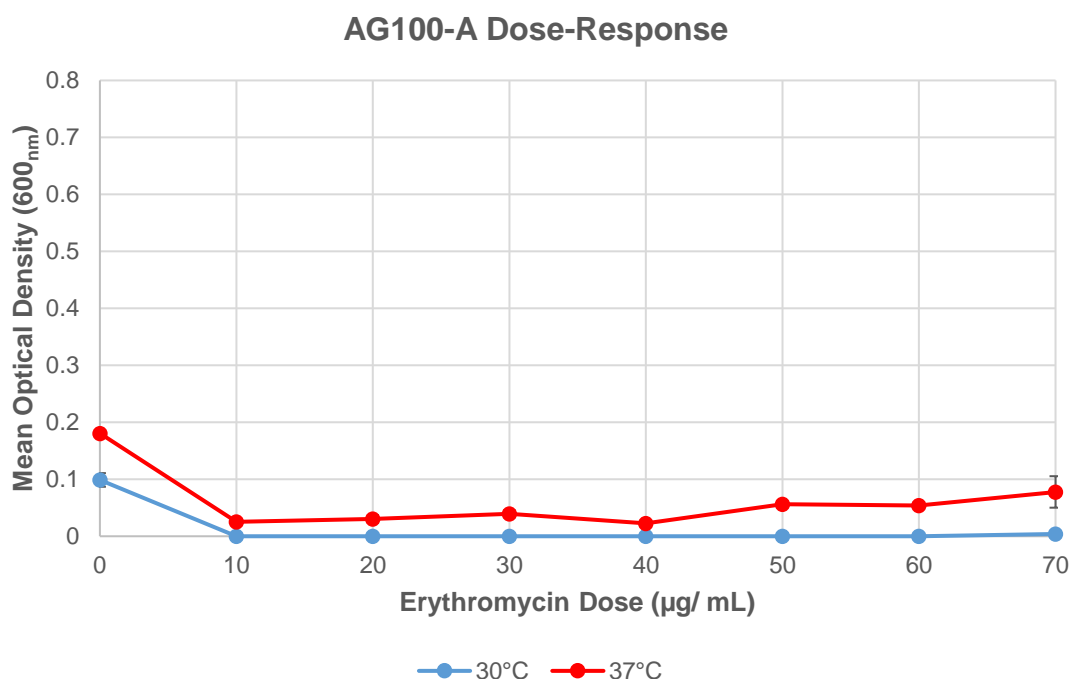
### 3.2.2 MIC Increases with Temperature

To investigate the effect of temperature on *E. coli* susceptibility to erythromycin, it was important to first establish the MIC of each strain at 30°C as reference.



**Figure 3.6:** Dose-response curves for *E. coli* strains MG1655 (WT, blue) and eTB108 (*acrB-sfGFP*, orange) at 30°C (circle) and 37°C (triangle). Wells of microplate containing erythromycin gradient and fresh M9 media inoculated with  $3.04 \times 10^6$  bacterial cells of MG1655 or eTB108. Inoculated plate then placed into plate reader at 30°C/ 37°C to read OD over 24 hours. Data points are average optical densities at the 24<sup>th</sup> hour and represent biological and technical triplicate, where error bars represent standard error of the mean. Error bars may be small and hidden behind data points.

At 30°C, according to Fig. 3.6, the point at which there is no visible growth i.e. the MIC occurs for both MG1655 and eTB108 between 20 – 30 µg/ mL. In contrast, *acrAB*-knockout strain AG100-A appears to be relatively more susceptible with an MIC between 0 – 10 µg/ mL (Fig. 3.7).



**Figure 3.7:** Dose-response curves for *E. coli* strain AG100-A ( $\Delta acrAB$ ) at 30°C (blue) and 37°C (red). Wells of microplate containing erythromycin gradient and fresh M9 media inoculated with  $3.04 \times 10^6$  bacterial cells. Inoculated plate then placed into plate reader at 30°C/ 37°C to read OD over 24 hours. Data points are average optical densities at the 24<sup>th</sup> hour and represent biological and technical triplicate, where error bars represent standard error of the mean. Error bars may be small and hidden behind data points.

With regards to temperature and its effect on the MIC, it appears that in both MG1655 and eTB108, there is a positive shift with increasing temperature (Fig. 3.6).

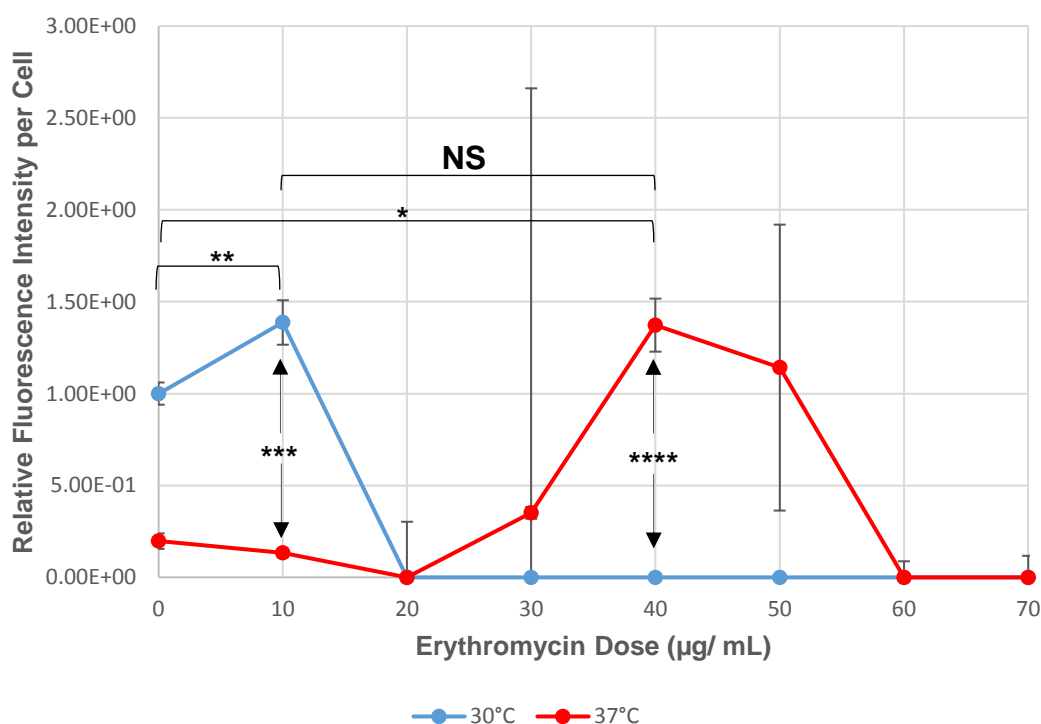
For MG1655, the MIC increases from 20 – 30 µg/ mL of erythromycin to 30 – 40 µg/ mL with increasing temperature.

The same trend of increase is seen in eTB108, however this time the shift occurs from an MIC of 20 – 30 µg/ mL at 30°C to 40 – 50 µg/ mL at 37°C – potentially a 2-fold increase from that of which is seen in MG1655.

However, with regards to AG100-A (Fig. 3.7), the effect of temperature on the MIC is inconclusive due to the level of noise at 37°C. From the initial part of the graph, it may be possible to predict no effect from temperature on MIC, however this experiment will need to be repeated to make any firm conclusions.

### 3.2.3 Selective Doses for Pump Expression Shift with Temperature

Using the FI data at the 24<sup>th</sup> hour recorded by the plate reader for eTB108 at both temperatures, pump expression levels per cell were calculated by initially blank-correcting the FI per dose and then obtaining an average from the biological and technical repeats. At the same time, to find the number of cells per dose, the mean OD for each antibiotic concentration was then multiplied by the mean OD-to-CFU/ mL conversion rate for eTB108 ( $1.57 \times 10^9$  or  $1.67 \times 10^9$  depending on the temperature of the FI data). The mean FI was then divided by the number of cells to give FI per cell values for each dose. However, to see how expression levels may vary with dose and temperature relative to the normal basal expression of stationary-phase eTB108, all values were then divided further by the average FI per cell value of eTB108 at 30°C with no erythromycin to provide relative values.



**Figure 3.8:** Relative expression levels of AcrB per cell as a function of erythromycin dose of sfGFP-tagged *E. coli* strain eTB108 (*acrB-sfGFP*) at 30°C (blue) and 37°C (red) at the 24<sup>th</sup> hour. FI per cell values for both temperatures are averaged and relative to basal expression of *acrB* at 24<sup>th</sup> hour at 30°C with no drug. Wells of microplate containing erythromycin gradient and fresh M9 media inoculated with  $3.04 \times 10^6$  bacterial cells. Inoculated plate then placed into plate reader at 30°C/ 37°C to read OD and FI over 24 hours. Data points represent average of biological and technical triplicate, where error bars represent standard error of the mean. Error bars may be small and hidden behind data points. Statistical significance using an unpaired t-test between two values where  $p < 0.05$  is represented by asterisk(s) (\*), and no statistical difference is denoted by 'NS' (NS:  $p = 0.9166$ , \*:  $p = 0.0308$ , \*\*:  $p = 0.0108$ , \*\*\*:  $p < 0.0001$ , \*\*\*\*:  $p < 0.0001$ ).

A visible difference can be seen in the peak of relative AcrB expression between the two experimental temperatures ([Fig 3.8](#)). At 30°C, the peak is at 10 µg/ mL of erythromycin at a value of ~1.39. At 37°C, a statistically similar peak can be seen at the dose of 40 µg/ mL. Both peaks of pump expression occur below their corresponding temperature-specific MIC (20 – 30 µg/ mL at 30°C and 40 – 50 µg/ mL at 37°C, as seen in [Fig 3.6](#)). In other words, selection for pump expression occurs at higher doses at the higher temperature.



### 3.3 Discussion

To reduce the rate at which antibiotic resistance is emerging, it is important to identify and study the factors that contribute to the rise, selection and fixation of resistance genes. Temperature, a potential selection pressure, has been recently shown to have a positive correlation with the percentage of bacteria, including *E. coli*, failing to respond to first-line antibiotics from a study that was conducted in the US [96]. However, how temperature selects for resistance mechanisms remains relatively underexplored. Understanding this cellular response is important, as ultimately treatments are designed and used to overcome these mechanisms specifically [20, 24, 25, 32, 131, 132, 133]. Although there are several mechanisms by which bacteria display resistance [7, 17], the aim of this chapter was to observe any changes in the susceptibility of *E. coli* to erythromycin as a result of temperature increase, and subsequently the effect of temperature on the selection of expression of MDR-associated AcrAB-TolC.

This was achieved by exposing all three strains of *E. coli* (MG1655, eTB108 and AG100-A) to a range of doses of erythromycin over 24 hours of growth at 30°C and 37°C. It was found that the erythromycin-associated MIC for both MG1655 and eTB108 increased as a result of increasing temperature (Fig. 3.6) and therefore the strains displayed a higher level of resistance at 37°C. In another study, an increase in resistance was also seen with *E. coli* and its response to rifampicin as a result of increasing temperature [108]. This was attributed to the selection and fixation of mutations within RNA polymerase, the target of rifampicin. Interestingly, in the aforementioned study, the *E. coli* cells were never exposed to rifampicin, concluding that the presence of the mutation provides an advantage at higher temperatures [108]. Another study showed that individual drugs respond differently based on the temperature that *E. coli* cells were adapted to [149]. Supporting the results in Fig. 3.6, the study found that erythromycin works synergistically with *E. coli* adapted to 30°C, i.e. a lower dose is required to inhibit bacterial growth, but antagonistically at 37°C. It was postulated (and shown experimentally for only the antibiotics tetracycline and

streptomycin) that antibiotics trigger HSPs and CSPs, similar to thermal stress [149]. This finding is valuable as it may help uncover similarities in the antibiotic and thermal response of pathogens, and by bridging this knowledge with how temperature selects for resistance mechanisms (such as drug efflux) can only further unshroud temperature-resistance relationships.

It should be noted that there was a greater increase in the MIC for eTB108 when shifting the temperature from 30°C to 37°C (Fig. 3.6), in comparison to MG1655. As mentioned in section 2.3, the evolution of TB108 to eTB108 via batch culture transfer may have allowed for adaptive mutations which increased the fitness of the strain. However, it may be speculated that other adaptive mutations have occurred, such as in other resistance (or resistance-regulatory) genes, which are expressed and generally more beneficial at 37°C (similar to RNA polymerase mutations and resistance to rifampicin as mentioned above [108]). The selection of such mutations may be in part due to the exposure of TB108 to a low concentration of erythromycin throughout the evolutionary experiment [80, 81]. As mentioned previously also in section 2.3, the development of non-efflux adaptations may be observed in real-time by exposing *acrAB*-knockout strain AG100-A to the conditions of the aforementioned evolutionary experiment - however this time the experiment to be conducted at 37°C. An increase in the carrying capacity over the duration of the experiment may indicate the presence of adaptive mutations.

The next experiment of this chapter measured the expression of AcrB in eTB108 as a response to temperature increase, via the fluorescence emitted from the sfGFP tag physically-fused to the *acrB* gene. Firstly, it was found that expression of the pump was selected for at sub-inhibitory concentrations (Fig. 3.8), which, in this discussion, will be termed as the AcrB-expression selection window. This is similar to results that have been observed in past studies, such as the selection of resistant mutants of *Salmonella* and *E. coli* with sub-MIC doses of other antibiotics [81], and even in the selection and maintenance of  $\beta$ -lactamase plasmid-carrying *E. coli* and *K. pneumoniae* cells [150]. In Fig. 3.8, interestingly, there is a positive shift in the AcrB-expression selection window when increasing the temperature from 30°C to 37°C, however the window is still maintained at concentrations below the 37°C-specific MIC. Why such a shift

occurs as a result of temperature change is difficult to explain with the lack of research on conditions that may affect the position of selective windows. Nonetheless, the implication of this result, i.e. the resistance-selective concentrations for certain antibiotics may change as a result of temperature, may prove useful for deciding the dose of a drug when treating infections in different parts of the world to different parts of the body.

An unexpected find is that in [Fig. 3.8](#) erythromycin, a translation inhibitor, is allowing the expression of the protein AcrB. In theory, due to the limited “free” ribosomes in the presence of erythromycin, the bacterial cell should not be able to translate even the protein components of further ribosomes let alone a proteinaceous efflux pump. The significance of this finding is that a clinical drug with a specific mode of action is not carrying out its intended function, but instead is potentially promoting the increase of resistance. Some theories suggest that ribosomes display selective protein synthesis when bound by antibiotic [\[151\]](#). Another theory suggests that there may be overexpression of ribosomal genes as a result of antibiotic exposure in order to dilute the effect of the drug molecules [\[152\]](#). These may explain the peaks of expression of AcrB ([Fig. 3.8](#)). Further work will need to be carried out on the effect of translational inhibitors on antibiotic resistance mechanisms which utilise proteins, in order to be able to continue using such drugs safely and effectively in the foreseeable future.

In chapter 2, although speculated, it was not possible to record any growth rate-yield trade-offs as a result of a lack of data around the time points where the exponential phase would have occurred. In this chapter, due to regular intermittent OD measurements over 24 hours, it was possible to show that the growth rate increased significantly from 30°C to 37°C for all *E. coli* strains ([Fig. 3.5](#)). However, trade-offs between growth rate and carrying capacity could not be revealed as the growth was continuous, and possibly even diauxic ([Fig. 3.2 - 3.4](#)). Diauxic growth normally occurs when two sugars are present in the medium and are successively metabolised, undergoing two growth cycles as a result [\[147\]](#). The minimal media used in these experiments contained only a limited amount of glucose (0.2% w/v) as the nutrient source, a common

concentration used to culture bacteria [153, 154, 155, 156]. Therefore diauxic growth should not have occurred. However, studies in the past have shown that casamino acids can also be used as a carbon source once the main nutrient has been depleted [157, 158]. This may provide an explanation for the continued growth seen in Fig. 3.2 – 3.4. Thus, there is a possibility that the growth curves shown in chapter 2 (Fig. 2.2 – 2.6) may also not have reached stationary phase. Therefore if this experiment were to be repeated, it should be carried out over a longer duration to make sure that stationary phase is achieved, using a nitrogen source that is less likely to be metabolised.

One of the questions that was posed during this research project was whether faster-growing *E. coli* cells are more susceptible to erythromycin than slower-growing *E. coli*, specifically when the growth rate is manipulated by temperature. Previous studies have shown that slow-growing bacteria may display a level of tolerance to sudden antibiotic exposure [102, 103, 105]. However, this was not the case in Fig. 3.6, where slower-growing *E. coli* cells were shown to be actually more susceptible to erythromycin. Furthermore, higher expression levels of AcrB were not seen in cells with lower growth rates, and the peak expression of the pump component was in fact statistically the same at both temperatures (Fig. 3.8), unlike previous studies that have shown enhanced efflux for slower-growing cells [102, 103]. However it should be noted that the evolution of slow-growers and fast-growers within a bacterial population is postulated to have occurred in order to overcome the scarcity of nutrients in the environment [138]. Therefore inducing differences in growth rate using temperature over a period of 24 hours is most likely to not yield the same results. However, in a long-term evolutionary experiment, it may be interesting to see how temperature facilitates tolerance.

## Chapter 4 – Concluding Remarks and Future Directions

Antibiotic resistance is emerging at an alarming rate all over the world [1, 2, 3]. Although the blame is usually placed on factors such as poor treatment behaviour [1, 2, 3], other conditions, including environmental, may potentially contribute to this increase in incidence of resistance [80, 83, 86, 87, 88]. This research project aimed to provide preliminary insights into the relationship of temperature and the selection of antibiotic resistance mechanisms, specifically the efflux pump AcrAB-TolC. This was done by exposing *E. coli* to 0 – 70 µg/mL of erythromycin at 30°C and 37°C and observing changes in the expression of AcrB, the antiporter component of AcrAB-TolC [53]. The results from these experiments show that an increase in temperature reduces the susceptibility of *E. coli* to erythromycin (Fig. 3.6), and that the concentrations at which selection of AcrB occurs also increase (Fig. 3.8). Ultimately, this supports the overall hypothesis of temperature being a potential selective pressure on antibiotic resistance. Furthermore, past studies along with the observations in this research project imply that the effect of temperature on drug susceptibility may be dependent on the strain and antibiotic being used [106, 107, 108, 149].

The temperatures 30°C and 37°C were originally chosen due to the range that exists around different parts of the body [92] and to mimic average seasonal temperatures seen around central latitudes such as China and India, where current literature has shown recent outbreaks of resistant infections [33, 98]. The implications of the findings from this research project may provide further insight in the way treatment can be tailored for certain temperatures to reduce the rate at which resistance is emerging e.g. one drug may be more effective and have less impact on resistance emergence when used on the skin or in a colder climate rather than for an internal infection of the body or in more temperate places. However, if this study were to be repeated, it may be useful to look at greater temperature ranges and their synergy with several classes of antibiotics against different pathogens, in order to pave the way to the possibility of temperature-dependent treatment of infections.

For future work, it may be beneficial to investigate how temperature may be involved specifically in the long-term evolution of antibiotic resistance. This

would involve looking at how quickly bacteria are able to adapt to re-exposure to antibiotic, where the adaptation rate can be defined by the change in growth rate over time [159]. From this it may be possible to determine at which temperatures adaptation occurs more quickly and at which doses for specific antibiotics, i.e. the presence of evolutionary hotspots [68].

Nonetheless, it is hoped that the findings of this research project provide a foundation into looking at environmental factors, such as temperature, and their selection on specific antibiotic resistance mechanisms.

## Supplementary

**Table S1:** *p*-values for [Fig. 2.2](#) (30°C growth curves) obtained from unpaired t-test.

Time (Hours)	<i>p</i> -values	
	MG1655 vs eTB108	MG1655 vs AG100-A
0	< 0.0001	< 0.0001
1	1	1
2	0.0163	0.0163
3	0.0096	0.0096
4	< 0.0001	< 0.0001
5	0.3046	0.012
6	0.0006	< 0.0001
7	< 0.0001	< 0.0001
8	0.7417	< 0.0001
24	0.57	< 0.0001
25	0.0087	< 0.0001
26	0.8318	< 0.0001
27	0.0087	< 0.0001
28	0.0001	< 0.0001
29	0.0014	< 0.0001
30	0.0069	< 0.0001
31	0.0004	< 0.0001

**Table S2:** *p*-values for [Fig. 2.3](#) (37°C growth curves) obtained from unpaired t-test.

Time (Hours)	<i>p</i> -values	
	MG1655 vs eTB108	MG1655 vs AG100-A
0	1	1
1	1	1
2	0.1284	0.05
3	< 0.0001	0.1284
4	0.0006	< 0.0001
5	0.0006	< 0.0001
6	< 0.0001	< 0.0001
7	< 0.0001	< 0.0001
8	< 0.0001	< 0.0001
24	0.1719	< 0.0001
25	0.0011	< 0.0001
26	0.1958	< 0.0001
27	0.0082	< 0.0001
28	0.0001	< 0.0001
29	0.0028	< 0.0001
30	0.7658	< 0.0001
31	0.0063	< 0.0001



**Table S3:** *p*-values for Fig. 2.4  
(MG1655 growth curves)  
obtained from unpaired t-test.

Time (Hours)	30°C vs 37°C <i>p</i> -values
0	< 0.0001
1	1
2	0.1423
3	< 0.0001
4	< 0.0001
5	< 0.0001
6	< 0.0001
7	< 0.0001
8	< 0.0001
24	0.0023
25	0.601
26	0.0046
27	0.1018
28	0.0321
29	0.0101
30	< 0.0001
31	0.0003

**Table S4:** *p*-values for Fig. 2.5 (eTB108 growth curves)  
obtained from unpaired t-test.

Time (Hours)	30°C vs 37°C <i>p</i> -values
0	1
1	1
2	< 0.0001
3	< 0.0001
4	< 0.0001
5	< 0.0001
6	< 0.0001
7	< 0.0001
8	< 0.0001
24	< 0.0001
25	< 0.0001
26	< 0.0001
27	< 0.0001
28	< 0.0001
29	< 0.0001
30	< 0.0001
31	< 0.0001

**Table S5:**  $p$ -values for Fig. 2.6 (AG100-A growth curves) obtained from unpaired t-test.

Time (Hours)	30°C vs 37°C $p$ -values
0	1
1	1
2	0.05
3	0.6032
4	1
5	1
6	0.0163
7	0.0008
8	0.0002
24	< 0.0001
25	< 0.0001
26	< 0.0001
27	< 0.0001
28	< 0.0001
29	< 0.0001
30	< 0.0001
31	< 0.0001

**Table S6:** *p*-values for Fig. 2.7 (MG1655 OD-to-CFU/ mL conversion factors) obtained from unpaired t-test.

Time (Hours)	30°C vs 37°C <i>p</i> -values
6	< 0.0001
8	< 0.0001
24	1
26	< 0.0001
28	< 0.0001
30	< 0.0001

**Table S7:** *p*-values for Fig. 2.8 (eTB108 OD-to-CFU/ mL conversion factors) obtained from unpaired t-test.

Time (Hours)	30°C vs 37°C <i>p</i> -values
6	< 0.0001
8	< 0.0001
24	1
26	1
28	1
30	< 0.0001

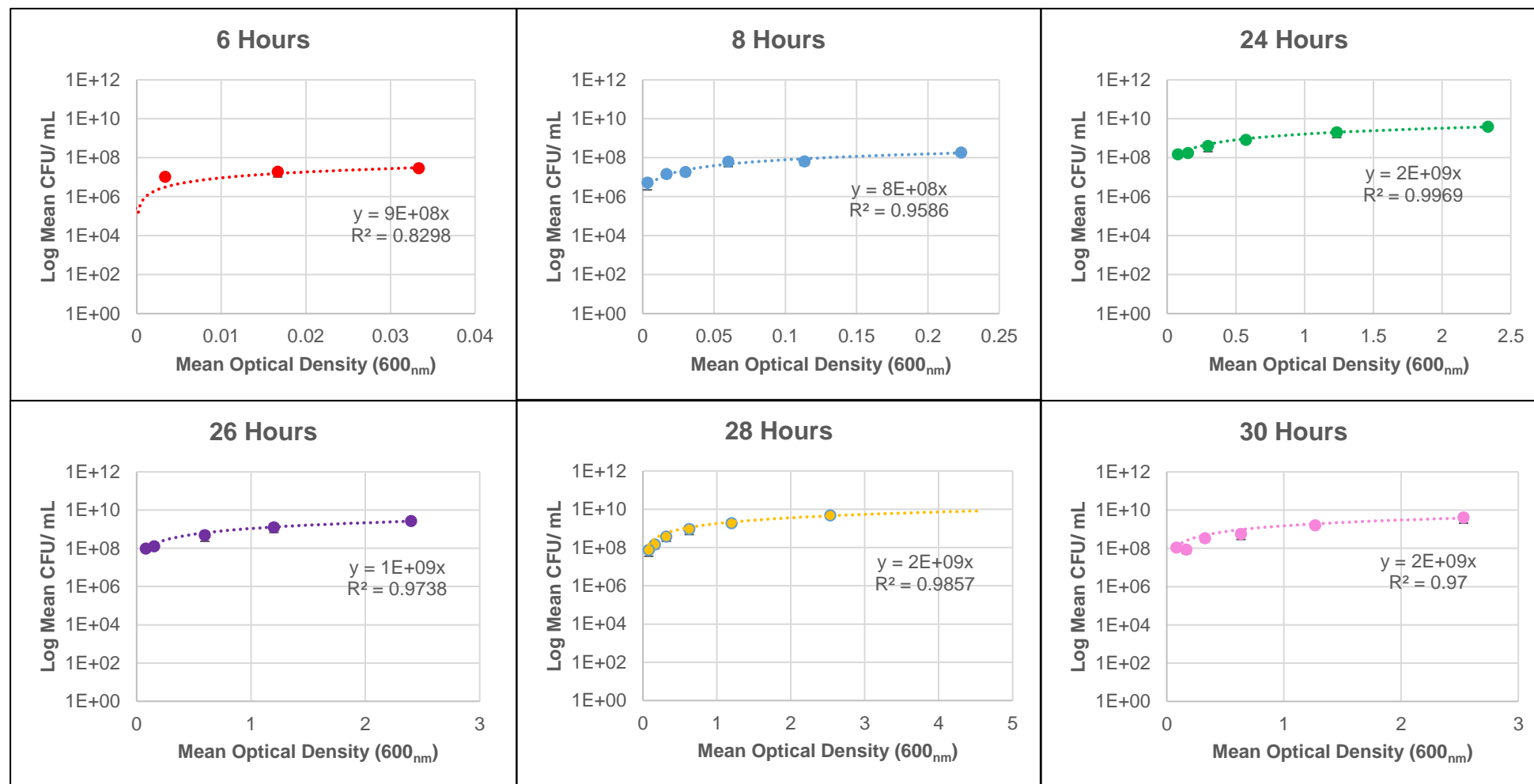
**Table S8:** *p*-values for Fig. 2.9 (AG100-A OD-to-CFU/ mL conversion factors) obtained from unpaired t-test.

Time (Hours)	30°C vs 37°C <i>p</i> -values
6	0.0035
8	0.0967
24	-
26	< 0.0001
28	< 0.0001
30	< 0.0001

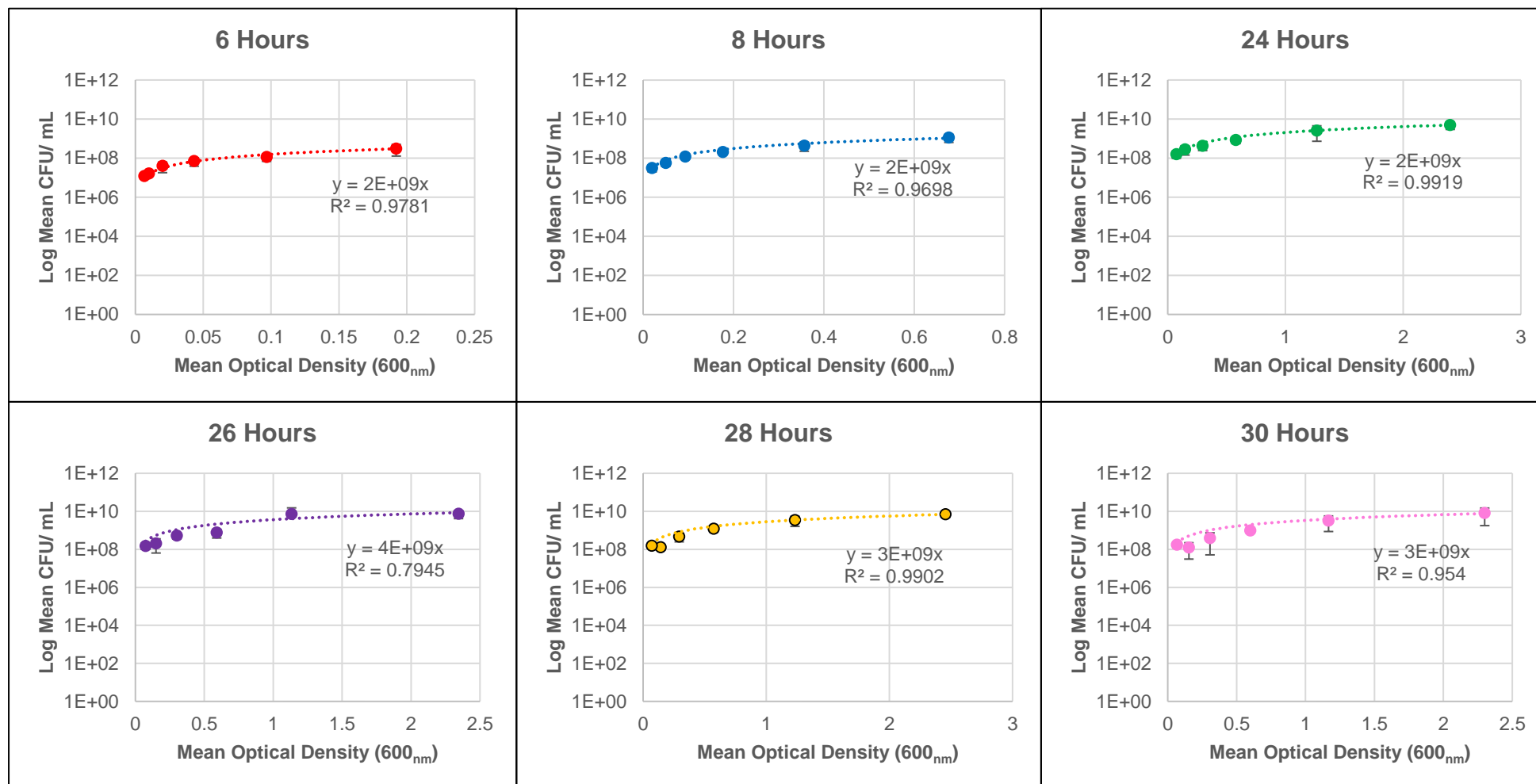
Note: data for the 24<sup>th</sup> hour omitted due to error

**Table S9:**  $p$ -values for [Fig. 2.10](#) (mean OD-to-CFU/ mL conversion factors) obtained from unpaired t-test.

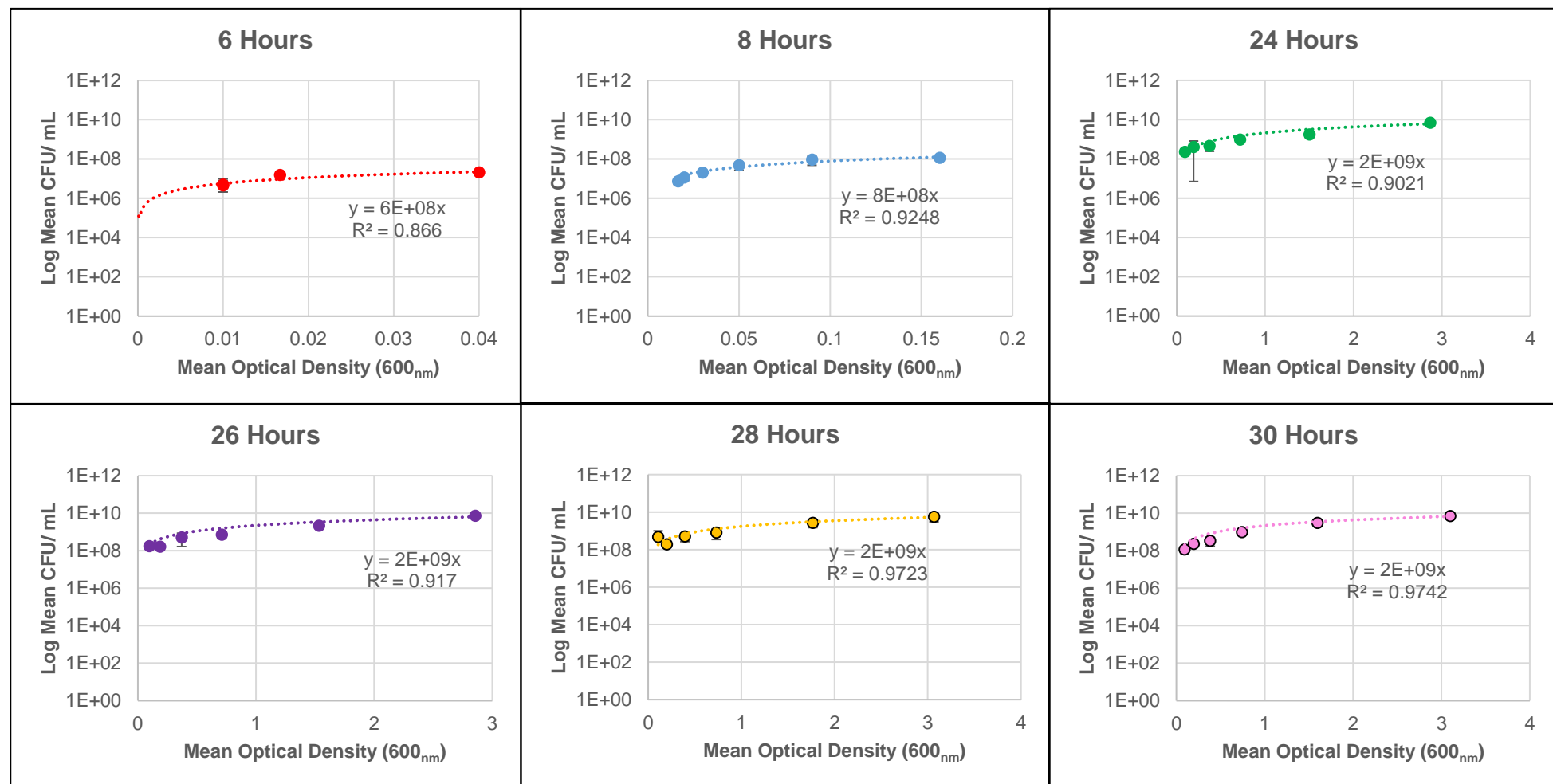
Mean OD-to-CFU/ mL Conversion Factor Comparison	$p$ -value
MG1655 30°C vs 37°C	0.0063
eTB108 30°C vs 37°C	0.7567
AG100-A 30°C vs 37°C	0.9915
eTB108 (30°C) vs MG1655 (30°C)	0.735
AG100-A (30°C) vs MG1655 (30°C)	0.0003



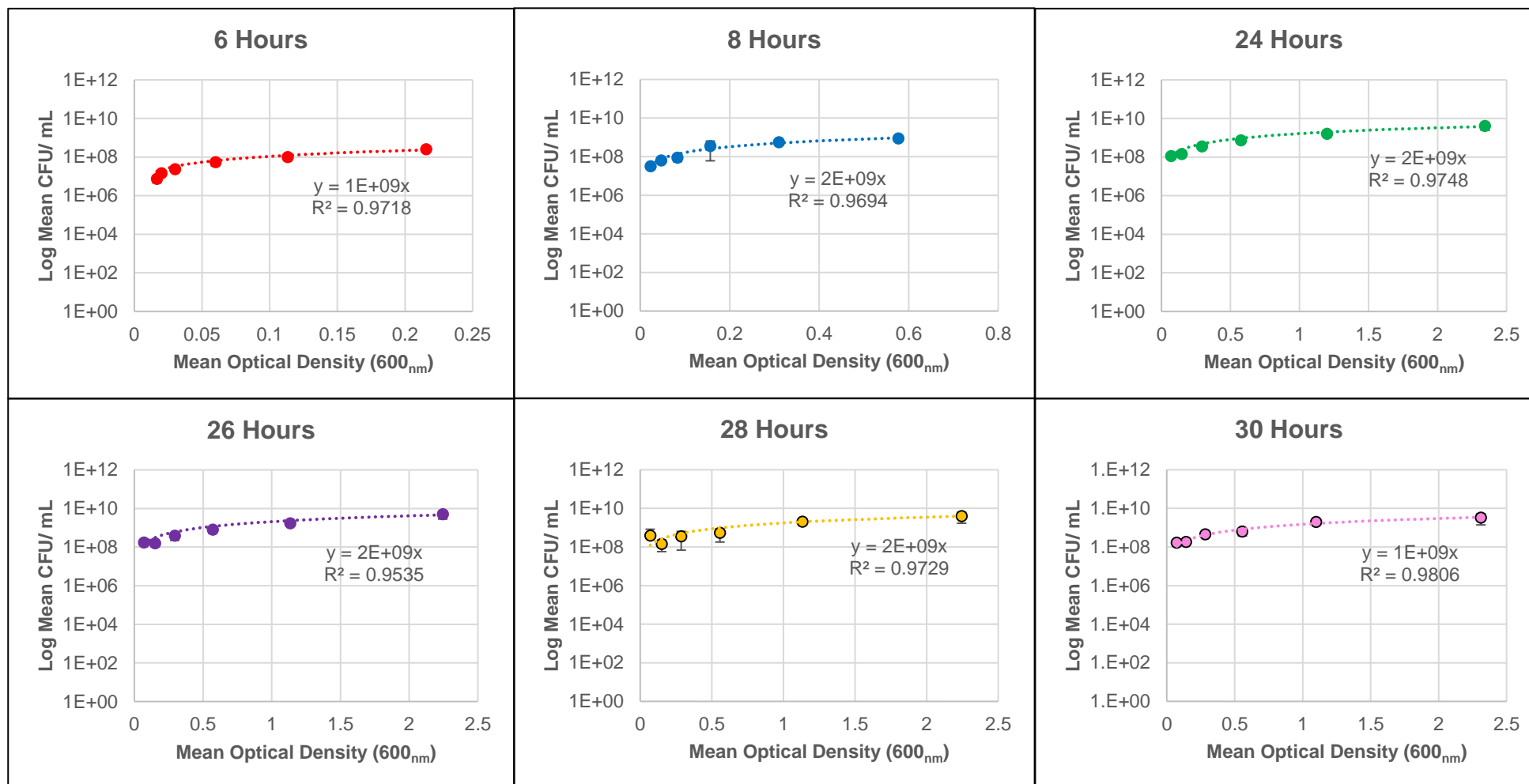
**Figure S1:** Regressions of mean OD vs log CFU/ mL for *E. coli* strain MG1655 (WT) at 30°C per time point. Overnight culture of each strain diluted 1:1000 and allowed to incubate for 31 hours. For hours 6, 8, 24, 26, 28 and 30, OD and CFU/ mL recorded for half dilutions to obtain equation of line i.e. OD-to-CFU/ mL conversion factors. Data points include biological and technical triplicate, where error bars represent standard error of the mean. Error bars may be small and hidden behind data points.



**Figure S2:** Regressions of mean OD vs log CFU/ mL for *E. coli* strain MG1655 (WT) at 37°C per time point. Overnight culture of each strain diluted 1:1000 and allowed to incubate for 31 hours. For hours 6, 8, 24, 26, 28 and 30, OD and CFU/ mL recorded for half dilutions to obtain equation of line i.e. OD-to-CFU/ mL conversion factors. Data points include biological and technical triplicate, where error bars represent standard error of the mean. Error bars may be small and hidden behind data points.

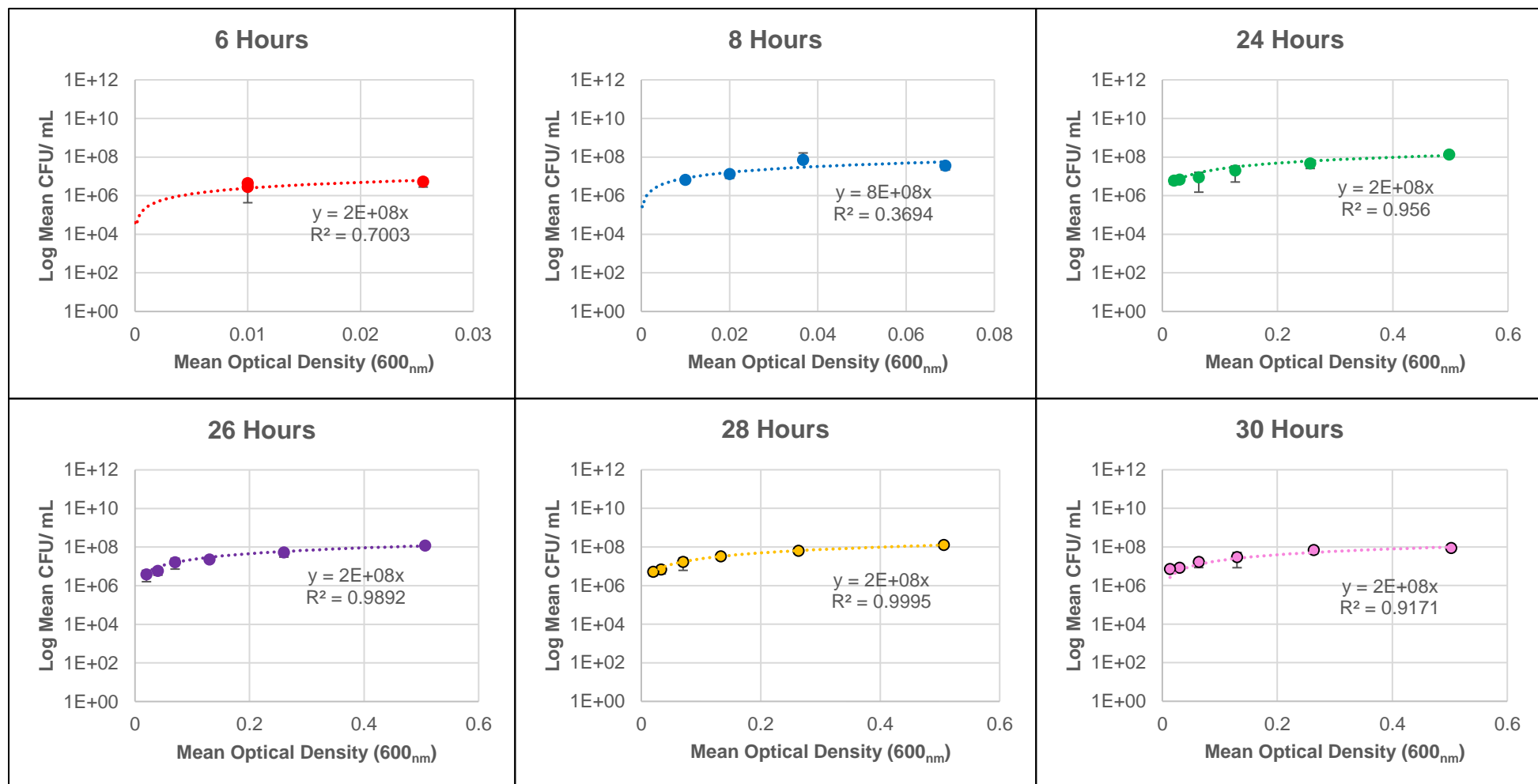


**Figure S3:** Regressions of mean OD vs log CFU/ mL for *E. coli* strain eTB108 (*acrB-sfGFP*) at 30°C per time point. Overnight culture of each strain diluted 1:1000 and allowed to incubate for 31 hours. For hours 6, 8, 24, 26, 28 and 30, OD and CFU/ mL recorded for half dilutions to obtain equation of line i.e. OD-to-CFU/ mL conversion factors. Data points include biological and technical triplicate, where error bars represent standard error of the mean. Error bars may be small and hidden behind data points.

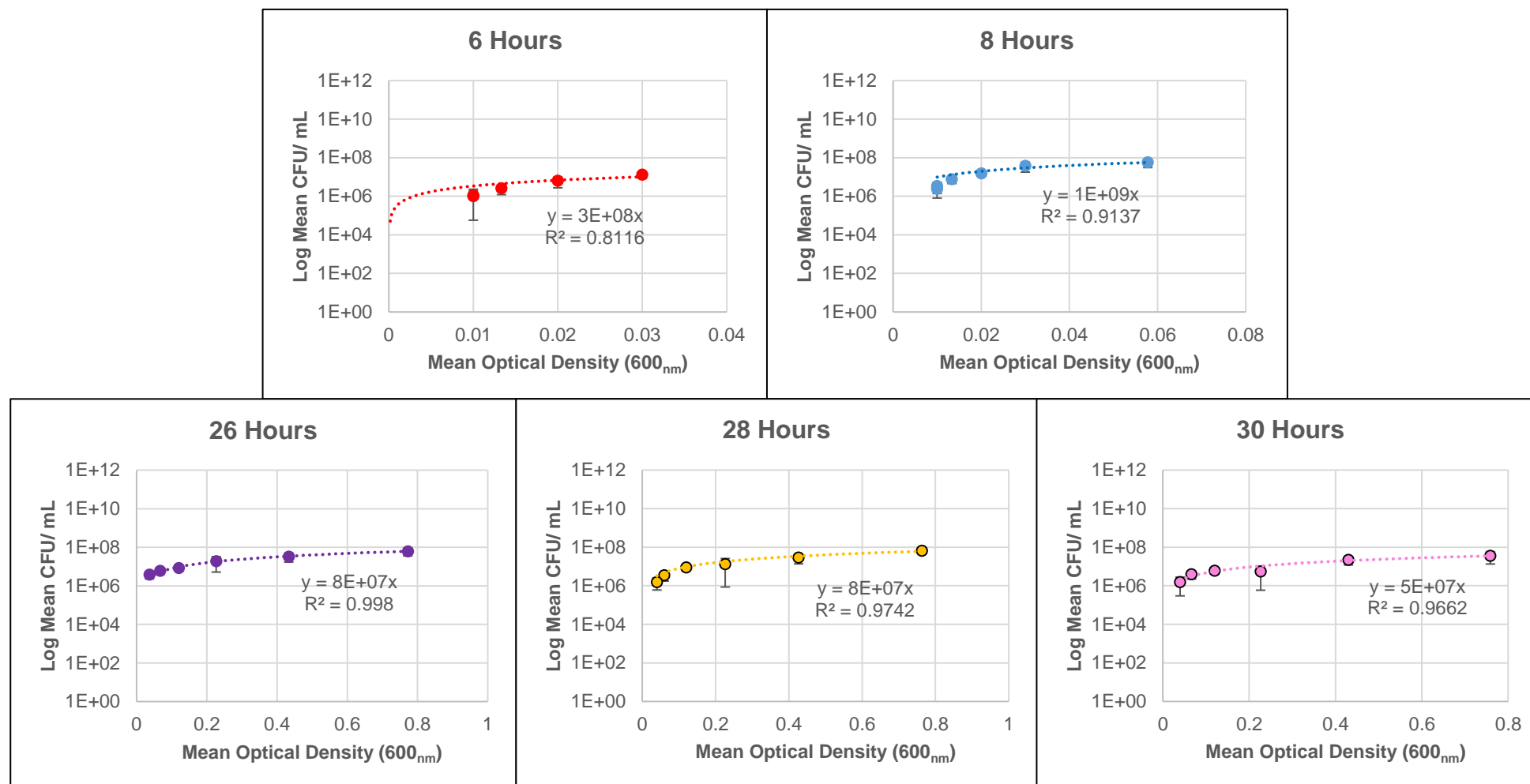


**Figure S4:** Regressions of mean OD vs log CFU/ mL for *E. coli* strain eTB108 (*acrB-sfGFP*) at 37°C per time point. Overnight culture of each strain diluted 1:1000 and allowed to incubate for 31 hours. For hours 6, 8, 24, 26, 28 and 30, OD and CFU/ mL recorded for half dilutions to obtain equation of line i.e. OD-to-CFU/ mL conversion factors. Data points include biological and technical triplicate, where error bars represent standard error of the mean. Error bars may be small and hidden behind data points.





**Figure S5:** Regressions of mean OD vs log CFU/ mL for *E. coli* strain AG100-A ( $\Delta acrAB$ ) at 30°C per time point. Overnight culture of each strain diluted 1:1000 and allowed to incubate for 31 hours. For hours 6, 8, 24, 26, 28 and 30, OD and CFU/ mL recorded for half dilutions to obtain equation of line i.e. OD-to-CFU/ mL conversion factors. Data points include biological and technical triplicate, where error bars represent standard error of the mean. Error bars may be small and hidden behind data points.



**Figure S6:** Regressions of mean OD vs log CFU/ mL for *E. coli* strain AG100-A ( $\Delta acrAB$ ) at 37°C per time point. Overnight culture of each strain diluted 1:1000 and allowed to incubate for 31 hours. For hours 6, 8, 26, 28 and 30, OD and CFU/ mL recorded for half dilutions to obtain equation of line i.e. OD-to-CFU/ mL conversion factors. Observations at 24 hours have been omitted due to error. Data points include biological and technical triplicate, where error bars represent standard error of the mean. Error bars may be small and hidden behind data points.

**Table S10:** OD-to-CFU/ mL conversion factors (CF) and  $R^2$  for each time point, per strain, per temperature

Time	MG1655				eTB108				AG100-A			
	30°C		37°C		30°C		37°C		30°C		37°C	
	CF (x 10 <sup>8</sup> )	$R^2$	CF (x 10 <sup>8</sup> )	$R^2$	CF (x 10 <sup>8</sup> )	$R^2$	CF (x 10 <sup>8</sup> )	$R^2$	CF (x 10 <sup>8</sup> )	$R^2$	CF (x 10 <sup>8</sup> )	$R^2$
6	9	0.8298	20	0.9781	6	0.866	10	0.9718	2	0.7003	3	0.8116
8	8	0.9586	20	0.9698	8	0.9248	20	0.9694	8	0.3694	10	0.9137
24	20	0.9969	20	0.9919	20	0.9021	20	0.9748	2	0.956	-	-
26	10	0.9738	40	0.7945	20	0.917	20	0.9535	2	0.9892	0.8	0.998
28	20	0.9857	30	0.9902	20	0.9723	20	0.9729	2	0.9995	0.8	0.9742
30	20	0.97	30	0.954	20	0.9742	10	0.9806	2	0.9171	0.5	0.9662

Note: data for AG100-A at 37°C at the 24<sup>th</sup> hour omitted due to error

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